Sulphite tolerance of yeasts from comminuted lamb products

Dillon, Vivian Maureen

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SULPHITE TOLERANCE OF YEASTS FROM COMMINUTED LAMB PRODUCTS

Submitted by Vivian Maureen Dillon

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

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SUMMARY

Species of Candida, Cryptococcus and Rhodotorula were recovered from pastures, fleece, carcass surfaces, minced lamb and lamb products. It was established that a route of yeast contamination extended from grazing areas through to lamb products. The numbers of Rhodotorula spp. varied seasonally in pastures and on fleece but remained consistently low on carcass surfaces and in meat products. Candida spp. dominated the yeast flora of sulphited or unsulphited minced lamb and lamb products.

Yeasts were a minor component (<5%) of the microbial contamination of pastures, fleece and carcass surfaces and remained so throughout the storage of preservative free minced lamb and lamb products. The addition of sulphite, however, favoured the growth of yeasts to such an extent that they dominated the microflora, until free sulphite diminished to a level which permitted growth of the sulphite sensitive Gram-negative bacteria particularly pseudomonads and Enterobacteriaceae.

The loss of free sulphite in meat products, and thereby preservative potential, was associated with an increase in bound sulphite resulting in part from acetaldehyde production by yeasts. Initially acetaldehyde producing strains dominated the yeast flora of minced lamb but, as the free sulphite level diminished, non-sulphite binding yeasts increased in number.

The sulphite tolerance of yeasts was shown to be determined by pH, sulphite concentration, substrate availability and the composition of the pre-incubation medium. Acetaldehyde production

by Candida norvegica was sulphite induced and occurred during the exponential phase of growth in sulphited (500 µg SO_2 ml^{-1}) lab lemco glucose broth cultures buffered at pH 5, 6 or 7. Growth was inhibited at pH 4 by sulphite concentrations >100 µg So_2 ml^{-1}. Acetaldehyde production also occurred in sulphited medium containing fructose or ethanol but not lactate. A non-acetaldehyde producing yeast, Candida vini, grew in sulphited (500 µg SO_2 ml^{-1}) lab lemco broth containing glucose or lactate buffered at pH 6 or 7 but not at pH 4 with <100 µg SO_2 ml^{-1} and at pH 5 with ≥250 µg SO_2 ml^{-1}.

The pH and glucose content of meat, particularly meat products with added carbohydrates, is ideal for acetaldehyde production by yeasts thus permitting a resultant increase in bound sulphite and concomitant decrease in the antimicrobial activity of the preservative.

INTRODUCTION

Although microbial spoilage of red meats has been studied almost since the beginnings of microbiology, it is only now that a comprehensive understanding of the physiological factors involved are starting to emerge. Many problems delayed progress. Until recently, for example, the inadequate classification of the principal spoilage organisms of chilled meats, Pseudomonas spp., impeded progress particularly in studies of the process of microbial colonisation and hence in the definition of factors that play an important role in selecting the spoilage flora. For many years also, an inadequate knowledge of the physiological basis for the differences between psychrotrophs and psychrophiles was another barrier to progress (Gounot, 1986). In spite of such problems, many investigators catalogued the contaminants of meat and the depots from which they came. These studies tended, however, to give too little attention to the diversity of habitats with which the live animal made contact and thereby the marked phylogenetic and hence physiological diversity of the flora carried to the slaughterhouse.

The first part of the literature review is concerned with the above aspects. It is evident that processing and storage methods of red meats impose selective pressures such that very few of the initial contaminants eventually flourish. Indeed it is now recognised that even with the organisms capable of causing spoilage subtle changes in processing or storage methods have profound effects on the selection of one organism at the expense of another. The review establishes also the cardinal role of glucose in the

selection and spoilage processes.

It is against a background of bacterial spoilage of meat that the limited and diffuse literature on yeasts is considered. Traditionally yeasts have played a minor role in meat spoilage because slow growth rates prevented effective competition with bacteria. Indeed, Walker and Ayres (1970) concluded that yeasts were unlikely to cause food spoilage unless bacterial competition was diminished. This is the case in the sulphited product, British fresh sausage (Dowdell and Board, 1971). Sulphite not only selects yeasts in this product, it also induces changes leading to loss of preservative activity in a manner analogous to that of yeasts in wines and ciders.

The advent of sulphited lamb products in the United Kingdom provided an opportunity to study: 1) the yeast infection routes of such products; 2) the role of sulphite in yeast selection in products containing mainly red meat; and 3) preservative loss with time. Two fundamentally important features were identified: 1) in meat glucose appears essential for sulphite binding; and 2) meat yeasts differ significantly from those of wines and ciders in their response to sulphite.

LITERATURE REVIEW

1. Microbial Contamination of Meat

Although yeast and fungal contamination of meat carcasses was discussed by Haines (1931) and Empey and Scott (1939), most of the research in the past half-century has been concerned with the numerically dominant bacteria. The latter are the predominant contaminants of stored meat because they outgrow yeasts and fungi (Mossel, 1971).

"Intrinsic" bacteria (Ingram, 1949) are found in the deep tissues of meat animals either before death or immediately after slaughter (Ayres, 1960b). Such contamination of healthy animals was assumed by some e.g. Ingram (1949) to result from the transfer by blood of organisms from the viscera. Ingram and Dainty (1971) reported that intrinsic contamination by this route, especially with mesophiles like Clostridium perfringens, led to rapid spoilage of meat stored under warm conditions and was responsible for "bone taint" of hams (Ingram, 1952). Gill (1979) disagreed with this interpretation. He concluded that bacteria did not pass across the intestinal wall or penetrate muscle tissue until several hours after death when tissue breakdown was well advanced and the rupture of the viscera released bacteria into the abdominal cavity (Gill et al., 1976; Gill and Penney, 1977; Gill, 1979). As under normal conditions the intact viscera is removed early in processing, breakdown will not occur. Indeed contamination from this source occurs only if the gut is punctured during evisceration (Empey and Scott, 1939; Newton et al., 1978).

Slaughtering instruments are probably the main cause of blood-borne contamination of deep tissues of the carcass. Mackey and Derrick (1979) inoculated instruments with genetically marked strains of Escherichia coli, Clostridium perfringens and Bacillus thuringiensis. Bacteria from the captive-bolt pistol were recovered from the spleen of beef cattle, and those from the pithing rod were found in the spleen and muscles of the flank and neck. Bacteria from the "stick" knife used to cut the throat of sheep, were found in the heart, lung, spleen, liver and kidneys. As most meat is stored under chill conditions, deep tissue contamination with mesophilic bacteria is of such little importance today that it can be concluded that the interior of the majority of commercially produced meat is effectively free of important spoilage bacteria (Gill, 1979; 1982).

Extrinsic bacteria - i.e. those originating from the environment (Ingram, 1949), on the other hand, are mainly psychrotrophic (Stokes and Redmond, 1966) and are commonly associated with meat spoilage. Indeed, the transfer of bacteria from the soil via the hides to the carcass and subsequently to the meat product has been well established.

The extrinsic bacterial contaminants of meat (Table 1) originate from diverse sources (Table 2) e.g. soil, water, vegetation and faecal material (Empey and Scott, 1939; Ingram and Dainty, 1971; Newton et al., 1978). They are brought into the slaughterhouse (Table 3) on hides, fleece, skin, feet, hooves and hair of animals (Empey and Scott, 1939; Ayres, 1955; 1960b; Ingram and Simonsen, 1980; McMeekin, 1982; Nottingham, 1982; Gill, 1986)

Table 1. Gram-negative bacteria from work surfaces, carcasses and meat at all stages of processing.*

Family or genus	Species	Number of Isolates
Pseudomonas	fluorescens	87
	putida	16
	fragi	26
	Unidentified	60
Enterobacteriaceae	Enterobacter aerogenes	55
	Serratia liquefaciens	59
	Erwinia caratovora	16
	Yersinia enterocolitica	12
	Klebsiella pneumoniae	9
	Hafnia alvei	5
	Citrobacter freundii	2
	Citrobacter amalonica	3
	Proteus rettgeri	3
	Serratia marcescens	14
Moraxella	Unidentified	96
Aeromonas	Unidentified	44
Acinetobacter	calocaceticus	25
Shewanella	putrefaciens**	23
Flavobacterium	Unidentified	19

* Adapted from Gill and Newton (1982)
** Formerly Alteromonas putrefaciens (MacDonell and Colwell, 1985)

Table 2. Bacteria isolated from carcass, meat and field samples

Organisms	References					
	1 §	2	3	4	5	6
Actinomyces spp.					+	
Aeromonas spp.			+			
Alcaligenes spp.					+	
Bacillus spp.					+	
Chromobacterium spp.					+	
Clostridium spp.					+	
Coryneforms		+	+			
Cytophaga spp.					+	
Enterobacter spp.					+	
Erwinia spp.	+					
Escherichia spp.					+	
Flavobacterium spp.	+		+		+	
Micrococcus spp.		+			+	
Pseudomonas spp.	+	+				
Moraxella spp.*			+		+	
Acinetobacter spp.*	+	+	+	+	+	
Salmonella spp.					+	
Brochothrix thermosphacta**						+

* Moraxella spp. and Acinetobacter spp. closely related to Pseudomonas spp. (Rossau et al., 1987).

** Brochothrix thermosphacta (Sneath and Jones, 1976) was formerly Microbacterium thermosphactum (McLean and Sulzbacher, 1953) isolated from pork sausages (Sulzbacher and McLean, 1951).

§ 1: Grass; 2: Hay - Druce and Thomas (1970);

3: Water - Jones (1973)

4: Water and soil - Warskow and Juni (1972);

5: Soil - Bryan (1980);

6: Soil and faeces - Gardner (1966)

Table 3. Bacteria isolated from fleece and hides.

Organisms	References					
	1§	2	3	4	5	6
Pseudomonas spp.	+			+		+
Acinetobacter spp.*				+		
Moraxella spp.*				+		
Brochothrix thermosphacta**			+			
Citrobacter spp.					+	
Coliforms					+	
Enterobacter spp.					+	
Enterobacteriaceae				+		
Escherichia spp.					+	
Klebsiella spp.					+	
Micrococci						+
Salmonella spp.		+				
Serratia spp.					+	
Staphylococci spp.						+

* Acinetobacter and Moraxella spp. closely related to Pseudomonas spp. (Rossau et al., 1987).

** Brochothrix thermosphacta formerly Microbacterium thermosphactum (Sneath and Jones, 1976)

§ 1: Fleece - Mulcock (1966); London and Griffith (1984).
2: Fleece - Grau and Smith (1974)
3: Fleece - Newton et al. (1978).
4: Hides - Newton et al. (1978)
5: Hides - Newton et al. (1977)
6: Hides - Ingram and Simonsen (1980)

in numbers ranging from $\log_{10}$ 1.28 - 5.66 cm^{-2} (Table 4). The microorganisms are easily transferred to the surface of the carcass during the removal of the hide and subsequent butchering operations (Tables 1, 4) *via* tools, work surfaces and hands (Empey and Scott, 1939; Kelly, 1978; Kelly *et al.*, 1982; Gill and Newton, 1982; Gill, 1986). The extent of such contamination often reflects the general hygiene of the slaughterhouse rather than the quality of equipment (Hudson *et al.*, 1987). A wash system designed to remove initial contaminants from carcasses at the end of the butchering process can make an important contribution to the shelf-life of meat (Anderson *et al.*, 1987).

A diverse range of bacteria have been isolated from freshly slaughtered animals (Table 1). As will be discussed subsequently, the storage conditions and chemical properties of meat favour the growth of very few of the initial contaminants. At the outset, chill storage plays an important role in that it elects psychrotrophic bacteria. These, which occur in natural environments and foods, have a maximum growth temperature above 20°C but can grow at 0°C (Gounot, 1986). Psychrophilic microorganisms, however, are restricted to permanently cold habitats and have a maximum growth temperature of 20°C or below (Gounot, 1986). Psychrophiles have thermolabile enzymes and exposure to high temperatures - sometimes as low as 13°C - initiates degradative activities resulting in lysis (Gounot, 1986) hence psychrophiles would not be expected to survive on the live animal or on the freshly butchered carcass.

Table 4. Microbial counts on hides, carcasses and meat* (log_{10} cm^{-2} - mean counts)

	BEEF								SHEEP				
	Hide		After dressing		After chilling		After boning		Fleece		After dressing		Combined meat counts
	T	P	T	P	T	P	T	P	T	P	T	P	(% P/T)
Summer	3.80	L	1.43	L	1.87	0.28	2.91	0.47	5.66	1.28	2.86	0.35	0.99
Autumn	4.28	1.87	1.91	0.40	2.12	0.64	3.08	1.09	5.11	2.36	2.45	0.25	1.65
Winter	4.62	2.54	1.91	0.99	2.01	1.37	2.75	1.43	5.45	3.29	2.92	1.20	7.43
Spring	NA	NA	2.15	1.18	1.44	0.20	2.75	0.43	5.41	2.22	2.94	1.61	3.36
Combined counts	4.34	1.78	1.85	0.56	1.96	0.68	2.92	0.88	5.38	2.40	2.78	0.77	
% P/T	0.27		5.09		5.24		0.91		0.11		0.98		

* Adapted from Newton et al. (1978)

T = Total counts

P = Psychrotrophic counts - determined by growth on nutrient agar after 2 weeks at 2°C

L = <1 cell cm^{-2} (log_{10} = <0.0)

NA = Samples not available

Yeast Contamination of Carcasses

When compared to bacteriological studies of meat, those concerned with yeasts have been few in number. The latter are reviewed in this section, the object being to establish whether or not the yeasts that originate in the field are transferred *via* the fleece to the lamb carcass and subsequently to a meat product. Many of the yeasts that occur in the field (Table 5) are psychrotrophic (do Carmo Sousa, 1969) and hence are potential spoilage organisms of meat under chill storage.

The yeast flora on pasture plants (rye grass and white clover) in New Zealand (Table 5) was dominated by *Bullera alba*, *Cryptococcus laurentii*, *Torulopsis ingeniosa*, *Rhodotorula glutinis*, *Rh. graminis*, *Rh. marina*, *Rh. mucilaginosa* and *Sporobolomyces roseus* (di Menna, 1959). The population size increased from 3.1×10^4 g^{-1} in December to 1×10^8 g^{-1} (wet wt) in March (di Menna, 1959). The percentage of pigmented yeasts (*Rhodotorula* spp. and *Sporobolomyces* spp.) decreased from 84-92% during February - March to 4-8% in August (di Menna, 1959).

The soil under pasture in New Zealand yielded a yeast flora (Table 5) dominated by *Cryptococcus albidus*, *Cr. terreus*, *Candida curvata* and *Schizoblastosporion starkeyi-henricii* (di Menna, 1957; 1960). The population size ranged from 6×10^3 to 2.4×10^5 yeasts g^{-1} soil wet wt (di Menna, 1957).

The soil from sheep yards in New Zealand (Baxter and Illston, 1977), on the other hand, was dominated by *Torulopsis candida* (3.4×10^3 c.f.u.g^{-1}) in association with *Cr. luteolus* (1×10^2 c.f.u.g.$^{-1}$) and *Rh. rubra* (1×10^2 c.f.u.g^{-1} wet wt).

Table 5. Yeast species isolated from soil, plants and air samples.

	References						
	§1	2	3	4	5	6	7
Bullera alba			+		+		
Candida albicans	+				+		
bovina			+				
curvata			+	+	+		
humicola			+	+	+		
lipolytica	+						
melinii						+	
parapsilosis	+						
rugosa			+				
scottii					+	+	
zeylanoides						+	
Cryptococcus albidus			+	+	+	+	
diffluens					+	+	
gastricus			+				
laurentii		+	+	+	+	+	
luteolus		+			+	+	
terreus			+	+			
Cryptococcus spp.							+
Debaryomyces kloeckeri	+		+			+	
marama						+	
nicotianae			+				
subglobosus	+					+	
Hanseniaspora vineae					+		
Hansenula candensis			+				
Kloeckera spp.							+
Pichia fermentans	+						
Rhodotorula crocea			+				
flava			+	+	+		
glutinis			+		+	+	
graminis			+		+		
macerans					+		
marina			+		+		
minuta	+		+			+	
mucilaginosa	+		+	+	+	+	
pallida						+	
rubra		+					
texensis			+		+		

Table 5. continued.

	References						
	1	2	3	4	5	6	7
Saccharomyces cerevisiae	+						+
Schizoblastosporion							
Starkeyi-henricii			+	+			
Sporobolomyces odorus					+	+	
pararoseus					+		
roseus			+		+	+	
salmonicolor						+	
Sporobolomyces spp.				+			
Torulopsis aeria				+			
candida		+	+			+	
dattila						+	
famata	+		+			+	
inconspicua			+				
ingeniosa			+		+		
Torulopsis spp.							+
Trichosporon cutaneum	+		+	+		+	
Trichosporon spp.						+	

§
1. Stockyard soils, New Zealand, di Menna (1955a).
2. Soil samples from sheep and cattle stockyards and paddocks, New Zealand, Baxter and Illston (1977).
3. Soil under pasture, New Zealand, di Menna (1960).
4. Leaves and roots of pasture grasses and herbs, New Zealand, di Menna (1957).
5. Pasture plants, New Zealand, di Menna (1958 a, b, c, 1959),
6. Airborne yeasts, New Zealand, di Menna (1955b).
7. Airborne yeasts, horticultural site, Canada, Adams (1964).

Rhodotorula mucilaginosa and C. parapsilosis were frequently found in stockyard soil (Table 5) in New Zealand by di Menna (1959).

Many yeasts are dispersed by air (do Carmo Sousa, 1969). Forty-two percent of the yeasts isolated from air in New Zealand were Cryptococcus spp., Debaryomyces spp. (26.2%) and Sporobolomyces-Rhodotorula spp. - 18.6% (di Menna, 1955b). More specifically, air samples from paddocks in New Zealand (Baxter and Illston, 1977) were dominated by T. candida and Rh. rubra (Table 6). The air near a fruit and vegetable site in Canada (Table 5) was similarly dominated by Cryptococcus spp., Torulopsis spp. and Kloeckera spp. (Adams, 1964).

Empey and Scott (1939) noted that, as compared to bacteria, a low proportion of yeasts and moulds were recovered from hides, soil and faeces (Table 7). In fact it can be computed from these results that yeasts accounted for only 0.02-0.22% at 20°C and 0.03-0.5% at -1°C of the microbial flora.

Psychrotrophic yeasts - Cr. laurentii, Cr. luteolus, Rh. rubra and T. candida - (Table 6) isolated from carcass and fleece (Baxter and Illston, 1976; 1977) were also evident in the field environment (Table 5) and in soil and air samples (Table 6) from paddocks (Baxter and Illston, 1977). Similarly, Rh. rubra and Cr. laurentii were isolated from sheep carcasses (Baxter and Illston, 1976). Lowry (1984), who studied lamb carcasses, noted that Candida spp. in association with Cryptococcus spp. predominated in Winter whereas Rh. rubra did so in Summer. He correlated these observations with those of di Menna (1959) who found similar seasonal changes with yeasts on pasture plants in New Zealand.

Table 6. Psychrotrophic yeasts associated with a sheep processing line*

	Cryptococcus laurentii	Cryptococcus luteolus	Candida guilliermondii	Rhodotorula rubra	Torulopsis candida
Stockyards/paddocks					
Soil	+	+	–	–	+
Air	–	–	–	+	+
Sheep pens					
Floor grating	–	–	+	+	–
Railings	–	–	–	+	+
Wool	–	+	–	+	+
Face	–	–	–	+	+
Slaughter area					
Carcass	+	–	–	+	–
Wall	–	–	–	–	–
Floor water	–	–	–	+	–
Bench top	–	–	–	+	+
Boning room					
Floor water	–	–	–	+	+
Air	–	–	–	–	+
Freezer					
Wall	–	–	–	–	+
Floor	–	–	–	+	+
Air	–	–	–	+	+
Chiller					
Bench	–	–	–	–	+
Wall	–	–	+	+	+

* Adapted from Baxter and Illston (1976, 1977)

Table 7. Microbial counts from contamination sources.*

Source of Contamination	Incubation Temperature (°C)	Bacteria	Yeasts	Moulds
Hides (cm^{-2})	20	3.3×10^{6}	5.8×10^{2}	8.5×10^{2}
	-1	1.5×10^{4}	3.9×10^{1}	8.9×10^{1}
Surface soil	20	1.1×10^{8}	5.0×10^{4}	1.2×10^{5}
(g^{-1} dry wt)	-1	2.8×10^{6}	1.4×10^{4}	1.0×10^{4}
Faeces	20	9.0×10^{7}	2.0×10^{5}	6.0×10^{4}
(g^{-1} dry wt)	-1	2.0×10^{5}	7.0×10^{1}	1.7×10^{3}
Rumen	20	5.3×10^{7}	1.8×10^{5}	1.6×10^{3}
(g^{-1} dry wt)	-1	5.2×10^{4}	$< 5.0 \times 10^{1}$	6.0×10^{1}
Air (in an abattoir)	20	1.4×10^{2}	-	0.2×10^{1}
(number deposited $cm^{2} h^{-1}$)	-1	0.8×10^{1}	-	1.0×10^{-1}

*Adapted from Empey and Scott (1939).

Contamination in the Abattoir

It is evident from the above that the live animal is the main means of introducing contaminants to the abattoir (Empey and Scott, 1939; Ayres, 1955). Abattoir equipment such as knives, work surfaces, cutting boards, boning tables, conveyors, carcass-washing water, slaughtermen's hands and aprons become directly contaminated from the fleece or by aerosols especially in the case of sheep having wet fleeces (Patterson, 1968b; Leach, 1971; Nottingham, 1982; Dainty et al., 1983).

Washing sheep before slaughter increased the moisture on the fleece and hence aided the spread of contaminants (Leach, 1971). Shearing before slaughter would reduce contamination of the carcass but the operation would not be cost effective because of the need for additional skilled labour (Leach, 1971). Chemical defleecing using cyclophosphamide (20-30 mg kg^{-1} body wt) is most effective in loosening the fleece if given 8 d prior to slaughter (Leach, 1971). This is impractical for sheep intended for slaughter. The bacterial load was reduced by this process but it needs to be stressed that sheep slaughtered only 24 h after dosing retained the toxic cyclophosphamide or its metabolites in the liver (Leach, 1971).

In an attempt to reduce contamination of carcasses, slaughtering areas are separated from the meat processing room (Ingram and Roberts, 1976). Footwear and protective clothing is made of impervious material, and knife sterilisers and hand-washing facilities are provided near the work station (Nottingham, 1982).

Candida guilliermondii, Rh. rubra and T. candida (Table 6) were recovered from walls, floors and benches in a freezing works

(Baxter and Illston, 1976). Rhodotorula rubra and T. candida also occurred on fleece and lamb carcasses (Table 6). Dalton (1984) isolated species of Cryptococcus, Rhodotorula, Torulopsis and Trichosporon from the slaughter area and the lairage in a pig processing plant.

Now that the use of wiping cloths and brushes are prohibited in the United Kingdom (Nottingham, 1982), carcasses are spray-washed. Patterson (1968b) observed that unwashed lamb carcasses had a bacterial count of $\log_{10}$ 6.49 which fell to $\log_{10}$ 6.30 (per total area of carcass) with a spray wash of 80°C. Using similar conditions, Kelly (1978) noted a reduction in initial bacterial numbers of $\geq \log_{10}$ 1.0 cm^{-2}. A further reduction of $\log_{10}$ 0.66 cm^{-2} was obtained when 30 µg ml^{-1} chlorine was included in the spray water. Increasing the chlorine concentration to 450 µg ml^{-1} only gave an additional reduction of $\log_{10}$ 0.29 cm^{-2} (Kelly, 1978; Kelly et al., 1981). Patterson (1968b) recommended the addition of 10 µg ml^{-1} chlorine to the spray in order to diminish the bacterial count of carcasses. It has been noted also that pathogens such as Staphylococcus aureus and Clostridium perfringens were reduced in number by spray washing (Kelly, 1978).

The spray-washed lamb carcasses were found, however, to have the same storage life as the unwashed ones (Kelly, 1978; Kelly et al., 1982). This was due to the number of bacteria (Pseudomonas spp. and Brochothrix thermosphacta) in the diaphragm region, where spoilage started, being little affected by the spray treatment. The Pseudomonas spp. and Br. thermosphacta accounted for less than 1% of the microflora after slaughter but their numbers increased to

$\log_{10}$ 6.12 - $\log_{10}$ 7.55 cm^{-2} when the carcass was deemed to have spoiled (Kelly et al., 1982). Ockerman et al. (1974) noted that 18% (v/v) acetic acid or 12% (v/v) lactic acid added to the spray wash reduced the number of microorganisms on lamb carcasses. Similarly, Anderson et al. (1987) found that the bacterial numbers were reduced on beef carcasses, machine-washed with 1.5% (v/v) acetic acid applied at 52°C.

At the end of a conventional processing line the lamb carcass (unsprayed) was found to have $\log_{10}$ 2.48 - 4.57 microorganisms cm^{-2} (Kelly, 1978). The major contaminants (isolated at 25°C) at this stage were Staphylococcus, Micrococcus and Flavobacterium spp. The psychrotrophic part of the bacterial flora was dominated by Moraxella, Pseudomonas and Acinetobacter spp. (Table 8). Further meat processing, however, selects a restricted range of yeasts (Deak and Beuchat, 1987). Candida, Cryptococcus and Rhodotorula spp. occurring in pastures (Table 5) and on fleece/carcasses (Table 6) were also isolated from meat products (Table 9).

It is evident from this review: (1) that even the best managed slaughter facilities do not prevent contamination of carcass meat; and (2) that yeasts are minor contaminants even though they are common contaminants of the environment in which animals are held before and at slaughter.

2. Meat as a Medium for Microbial Growth

Meat is an ideal medium for microbial growth. It has an optimum pH (5.8 - 6.8), a high water content (a_w = 0.99), a rich supply of nitrogenous substances and a depot of carbohydrates and essential

Table 8. Occurrence of microorganisms on freshly slaughtered lamb carcasses.*

Organisms	Isolated at 25°C (%)	Psychrotrophic (%)
Staphylococcus spp.	20	1
Micrococcus spp.	16	6
Flavobacterium spp.	14	9.5
Pseudomonas spp.	9.5	22
Moraxella spp.**	3	40
Acinetobacter spp. **	2	20
Bacillus spp.	8	
Brochothrix thermosphacta+	4	1
Enterobacteriaceae	2	
Vibrio or Aeromonas spp.	1	
Streptococcus spp.	0.5	
Unclassified:		
Yeasts	0.5	
Actinomycetes	5	
Gram +ve rods	11	0.5
Gram -ve rods	2	0.5
Gram variable coccobacilli	0.5	

* Adapted from Kelly (1978).

** Moraxella spp. and Acinetobacter spp. closely related to Pseudomonas spp. (Rossau et al., 1987).

\+ Formerly Microbacterium thermosphactum (Sneath and Jones, 1976).

Table 9. Yeast species isolated from meat and meat products.

	References[§]
Brettanomyces spp.	5
Bullera alba	23
tsugae	23
Candida spp.	4, 5, 11, 22, 28
Candida albicans	17, 23
blankii	2
brumptii	13, 14
buffonii	2
catenulata	11
ciferrii	23
curvata	2, 23
diddensiae	2
diversa	2
famata	3
foliorum	23
glaebosa	2
guilliermondii	14, 24, 29
humicola	23, 27, 29
iberica	2, 15
ingens	23
insectamans	2
intermedia	2, 26
krusei	26
lambica	2
lipolytica	2, 3, 11, 13, 14, 16, 17, 19, 28, 29
lipolytica var. deformans	23
lipolytica var. lipolytica	23
melinii	16
mesenterica	23
parapsilosis	3, 14, 17, 19, 20, 21, 24, 28, 29
pelliculosa	26
ravautii	23
rugosa	13, 14, 16, 23, 26
sake	3, 23

Table 9. continued

	References[§]
Candida scotti	26, 27
silvae	23
silvicultrix	2
steatolytica	2
tropicalis	17, 21
valida	23
vini	23
zeylanoides	2, 3, 6, 11, 16, 17, 19, 23, 28, 29
Cryptococcus spp.	4, 5
Cryptococcus albidus	27, 19
albidus var. aerius	23
albidus var. albidus	3, 23
albidus var. diffluens	23
diffluens	27, 28
dimennae	23
gastricus	23
hungaricus	23
infirmo-minatus	3, 6, 29
laurentii	2, 19, 27, 29
laurentii var. flavescens	23
laurentii var. laurentiii	3, 6, 23
laurentii var. magnus	23
luteolus	25
macerans	23, 29
neoformans var. uniguttulatus	27
skinneri	23
uniguttulatus	23
Debaryomyces spp.	4, 5, 7, 10, 20, 22
Debaryomyces hansenii	7, 8, 9, 11, 13, 14, 18, 20, 23
kloeckeri	7, 9, 11, 13, 14, 16, 20, 28
marama	23
nicotianae	11, 13, 14, 20
subglobosus	11, 13, 14, 16, 28

Table 9. continued.

	References [§]
Geotrichum candidum	24
Hansenula spp.	20
Hansenula anomala	17
Hansenula anomala polymorpha	23
Hansenula subpelliculosa	16
Leucosporidium capsuligenum	23
Leucosporidium scotti	23
Pichia spp.	4
Pichia etchellsii	23
Pichia farinosa	17
Pichia media	23
Pichia membranaefaciens	23
Pichia vini var. melibiosi	23
Pichia vini var. vini	23
Rhodotorula spp.	4, 5, 6, 12, 20
Rhodotorula aurantiaca	25, 26
Rhodotorula flava	25
Rhodotorula glutinis	2, 6, 16, 19, 23, 25, 27, 29
Rhodotorula glutinis var. rubescens	28
Rhodotorula graminis	23
Rhodotorula marina	2, 23
Rhodotorula minuta	24, 25, 27, 28
Rhodotorula minuta var. minuta	23
Rhodotorula minuta var. texensis	3
Rhodotorula mucilaginosa	16, 26, 27, 28
Rhodotorula pallida	23, 29
Rhodotorula rubra	2, 3, 17, 19, 20, 23, 28, 29, 30
Saccharomyces acidifaciens	16
Saccharomyces cerevisiae	24
Saccharomyces dairensis	24
Saccharomyces kloeckerianus	14, 20

Table 9. continued

	References [§]
Sporobolomyces spp.	5
Torulopsis spp.	5, 20, 22
Torulopsis aeria	16
albida	24
candida	2, 11, 16, 17, 18, 19, 20, 23, 25, 27, 28
domercqii	23
famata	13, 14, 25, 26, 27, 28
glabrata	26
globosa	24, 27
Torulopsis gropengiesseri	11
holmii	24
inconspicua	23, 26
inconspicua var. inconspicua	28
norvegica	23
pseudaeria	28
silvatica	2
vanderwaltii	23
versatilis	2, 16, 23
Trichosporon spp.	4, 5, 22, 27, 30
Trichosporon cutaneum	2, 3, 19, 20, 23, 29
pullulans	2, 3, 6, 11, 16, 23, 26, 27, 29
variable	29

[§] 1 Beef; Ayres (1960a)
2 Ground beef; Hseih and Jay (1984)
3 Minced beef radurised; Johannsen et al. (1984)
4 Minced beef; Nychas (1984)
5 Pork meat; Szczepaniak et al. (1975)
6 Lamb; Lowry (1984), Lowry and Gill (1984)
7 Sausages; Cesari (1919), Cesari and Guilliermond (1920)
8 Weiner sausages; Mrak and Bonar (1938)
9 Meat brines; Costilow et al. (1954)
10 Luncheon meats; Wickerham (1957)
11 Frankfurters; Drake et al. (1958, 1959)
12 Dried beef; Frazier (1958)
13 Cured meats; Bem and Leistner (1970)
14 Cured meat, ham, sausages; Leistner and Bem (1970)
15 Spanish sausages; Ramírez and González (1972)

Table 9. continued

16	Sausages, savoloy, meat, black puddings, pies; Aboukheir and Kilbertus (1974)
17	Tea sausages; Živanović and Ristić (1974)
18	Cured meats; Smith and Hadlok (1976)
19	Sausages; Abbiss (1978)
20	Dry sausages, Comi and Cantoni (1980a, 1980b)
21	Sausage, ham; Staib et al. (1980)
22	Sausages; Banks (1983)
23	Sausages; Dalton (1984)
24	Chicken; Njoku-Obi et al. (1957)
25	Poultry; Wells and Stadelman (1958)
26	Poultry; Walker and Ayres (1959)
27	Crab meat; Eklund et al. (1965, 1966)
28	Marine fish; Ross and Morris (1965)
29	Sea urchin, tuna; Kobatake and Kurata (1980)
30	Shellfish; Hood (1983)

growth factors, such as minerals and vitamins (Table 10). Under normal conditions of meat processing and storage, bacteria are the principal exploiters of this "medium".

The ultimate pH of muscle varies from 5.5 to 7.0 depending on the quantity of glycogen available for conversion to lactic acid during anaerobic glycolysis (Gill and Newton, 1978, 1982; Lawrie, 1985). Enterobacter spp., Br. thermosphacta, non-fluorescent and fluorescent Pseudomonas spp. grow at maximum rates at pH values between 5.5 and 7.0 (Gill and Newton, 1977). The growth rate of Acinetobacter, however, is reduced at pH 5.7 and below (Gill and Newton, 1977). Bacterial proteases are active at a pH near neutrality, whereas carbohydrases have an optima below pH 6 and operate as low as pH 5.5 in the lactic acid bacteria (Lawrie, 1985). At a pH below 5.5, bacteria are inhibited and acid-tolerant yeasts grow (Jay, 1978, 1979). Thus in meat products fermented by lactic acid bacteria, such as thuringer, Summer sausage, pepperoni, cervelat and Genoa salami (Johnston and Elliott, 1976) the low pH permits growth of yeasts.

Gram-negative bacteria tolerate an a_w of 0.94 - 0.97 (Scott, 1957; Troller and Christian, 1978). The reduced water activity of processed meats (Jay, 1978, 1979; Beuchat, 1983) and cured meats (Bem and Leistner, 1970) is therefore selective for yeasts that grow at an a_w of 0.62 - 0.98 (Corry, 1978).

The main components of muscle (Table 10); protein and fat, do not alter appreciably in composition during rigor development. Moreover, bacteria do not breakdown proteins or fats until other substrates are depleted (Dainty et al., 1975; Gill and Newton,

Table 10. Chemical composition of typical mammalian muscle after *rigor mortis* and before degradative changes *post mortem*.*

Components		Wet weight (%)
1. Water		75.00
2. Protein		19.00
(a) Myofibrial	11.5	
(b) Sacroplasmic	5.5	
(c) Connective tissue and organelles	2.0	
3. Lipid		2.50
neutral lipid, phospholipids, fatty acids, fat-soluble substances	2.5	
4. Carbohydrates		1.20
lactic acid	0.90	
glucose-6-phosphate	0.15	
glycogen	0.10	
glucose, traces of other glycolytic intermediates	0.05	
5. Miscellaneous soluble non-protein substances		2.30
(a) nitrogenous	1.65	
(b) inorganic	0.65	
6. Vitamins		

* Adapted from Lawrie (1985)

1981). Indeed their growth on meat is at the expense of low molecular weight soluble components (Gill, 1986). The concentrations of these alter during development of *rigor* (Table 11) resulting from the degradation of glycogen *via* glycolysis (Gill, 1982; 1986).

Anaerobic Glycolysis

The glycogen level is the main determinant controlling the fall in adenosine triphosphate (ATP) concentration and hence the length of time between death and the onset of *rigor mortis* (Eskin *et al.*, 1971). In well fed and rested animals the glycogen is at a maximum level and a long interval before *rigor* produces good quality meat with an ultimate pH of 5.3 to 5.5 (Eskin *et al.*, 1971). If, however, the animal undergoes stress, starvation, exercise or struggles prior to slaughter, then the glycogen level is depleted (Bate-Smith and Bendall, 1949; Eskin *et al.*, 1971; Pearson *et al.*, 1974; Tarrant, 1976; Gill, 1982; Lawrie, 1985) and the onset of *rigor* is rapid causing an inferior meat product with a pH of 6.0 to 6.6 (Eskin *et al.*, 1971; Gill, 1982). The term, DFD, is given to the resultant dark, firm, dry meat (Newton and Gill, 1978a).

Adenosine triphosphate concentration is maintained in the living muscle by aerobic oxidation of glycogen *via* pyruvate and the tricarboxylic acid (TCA) cycle to carbon dioxide and water (Fig. 1). The accompanying resynthesis of ATP from adenosine diphosphate (ADP), oxidative phosphorylation, *via* the electron transport chain produces 36 ATP per glucose residue in glycogen (Gill, 1982; Jeacooke, 1984). At death, oxidative metabolism

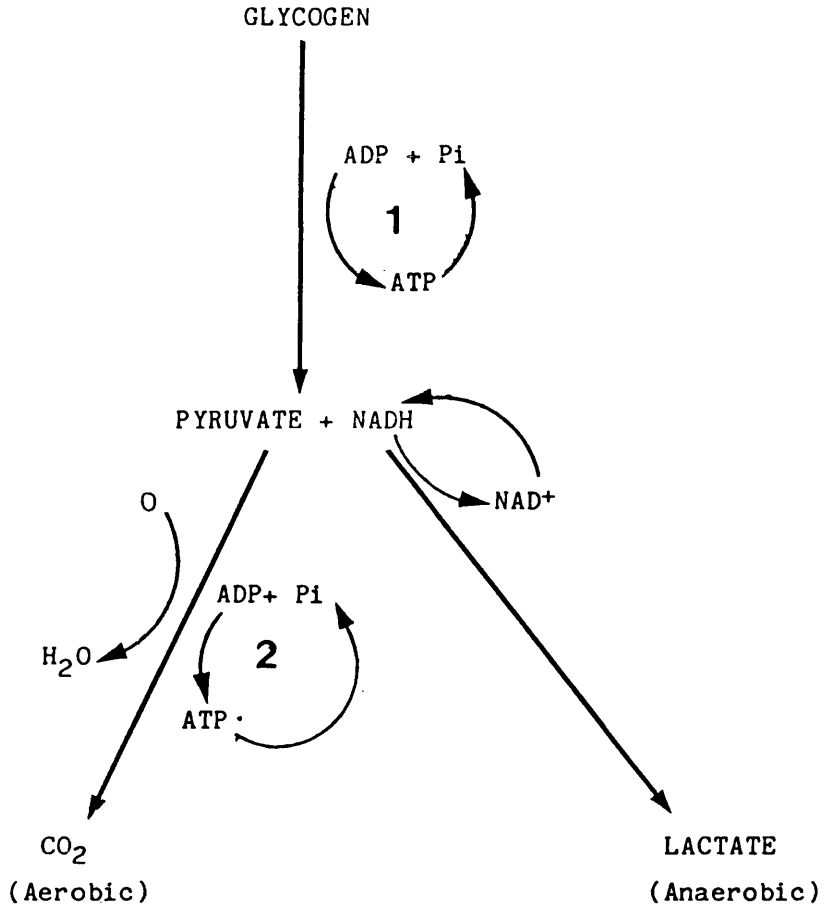
Table 11. Concentration of the main low molecular weight soluble components of beef before and after completion of rigor*

Substance	Concentration mg g^{-1}	
	Prerigor	Postrigor
Creatine phosphate	3.0	-
Creatine	4.5	6.5
Adenosine triphosphate	3.0	-
Inosine monophosphate	0.2	3.0
Glycogen	10.0	1.0
Glucose	0.5	0.1
Glucose-6-phosphate	1.0	0.2
Lactic acid	1.0	9.0
Amino acids	2.0	3.5
Dipeptides (carnosine anserine)	3.0	3.0
pH	7.2	5.5

*Adapted from Gill (1982, 1986).

Figure 1.

AEROBIC AND ANAEROBIC METABOLISM OF MUSCLE GLYCOGEN *



3 Cr P + ADP $\rightleftharpoons$ Cr + ATP

1. Substrate level phosphorylation.
2. Oxidative phosphorylation.
3. ATP resynthesis at the expense of conversion of creatine phosphate to creatine.

* Adapted from Jeacooke, 1984.

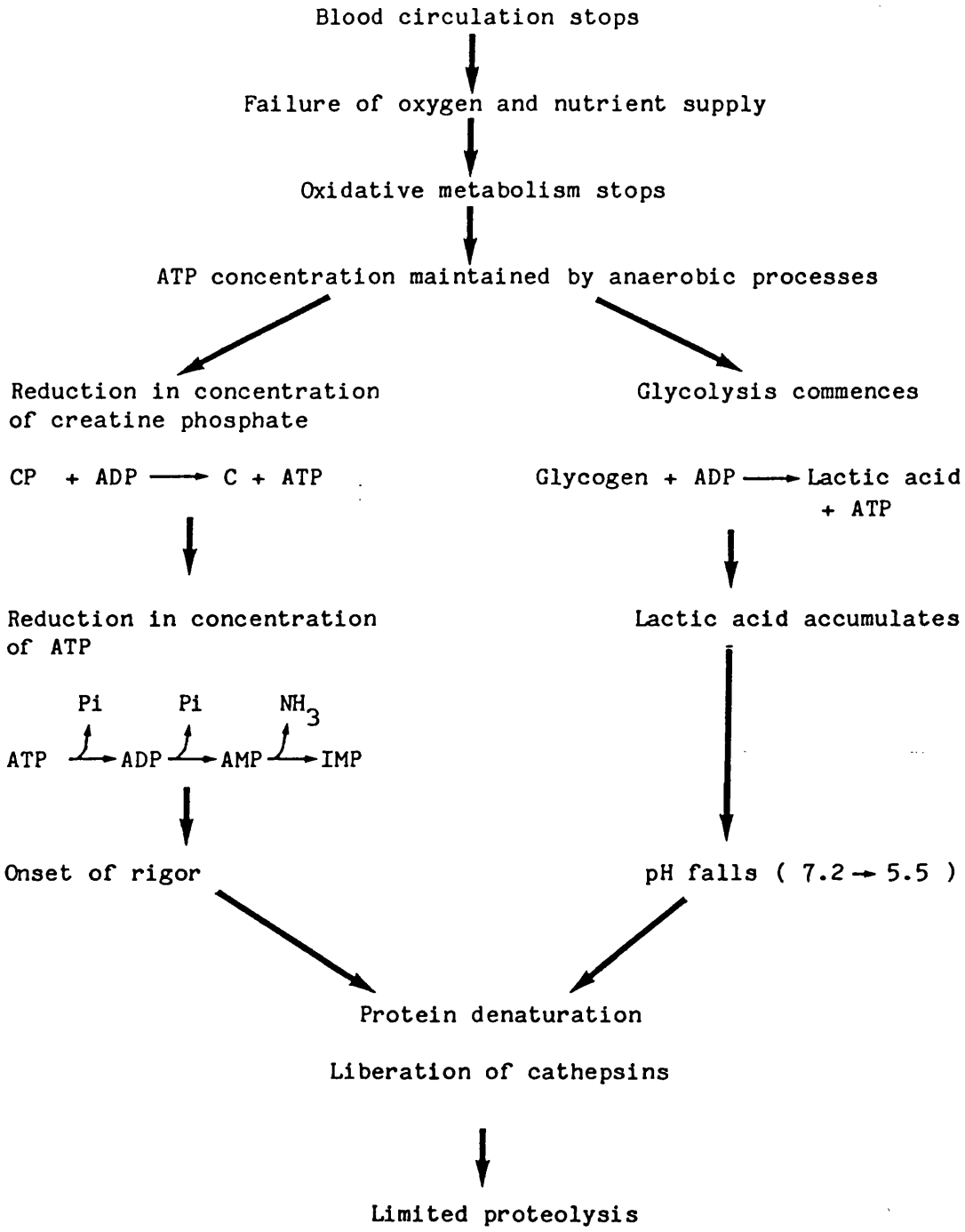
ceases, and ATP resynthesis is dependent on anaerobic processes (Jeacooke, 1984). The ATP level is initially maintained by resynthesis of ADP (Fig. 2) and creatine phosphate -CP- (Gill, 1982; Jeacooke, 1984; Lawrie, 1985; Coulson, 1987) when these are depleted glycogen is the major source of ATP resynthesis (Jeacooke, 1984; Lawrie, 1985). There are two possible pathways for anaerobic degradation of glycogen (Fig. 3). The first, the hydrolytic or amylolytic pathway produces glucose from glycogen and is important in fish muscle (Eskin et al., 1971). The process by which glucose is formed in mammalian muscle remains speculative (Bendall, 1973). The second pathway is the main one in mammalian muscle (Eskin et al., 1971). Glycogen is degraded to lactic acid via anaerobic glycolysis with the production of only 3 ATP per glucose residue in glycogen by substrate level phosphorylation (Fig. 1) of ADP (Jeacooke, 1984; Coulson, 1987).

The maintenance of the ATP level against continual ATPase activity eventually depletes the concentrations of creatine phosphate and glycogen with the resultant fall in ATP (Table 11) and increase in inosine monophosphate (IMP). The ADP that accumulates is degraded to adenosine monophosphate (AMP) which is deaminated to IMP and ammonia (Bendall and Davey, 1957; Gill, 1982, 1986).

The accumulation of lactic acid causes a fall in pH (Table 11) from 7.2 to 5.5 (Bate-Smith and Bendall, 1949; Newbold and Lee, 1965; Newbold and Scopes, 1967, 1971; Eskin et al., 1971). At this pH the glycolytic enzymes are inactivated (Bate-Smith and Bendall, 1949; Lawrie, 1985).

Figure 2

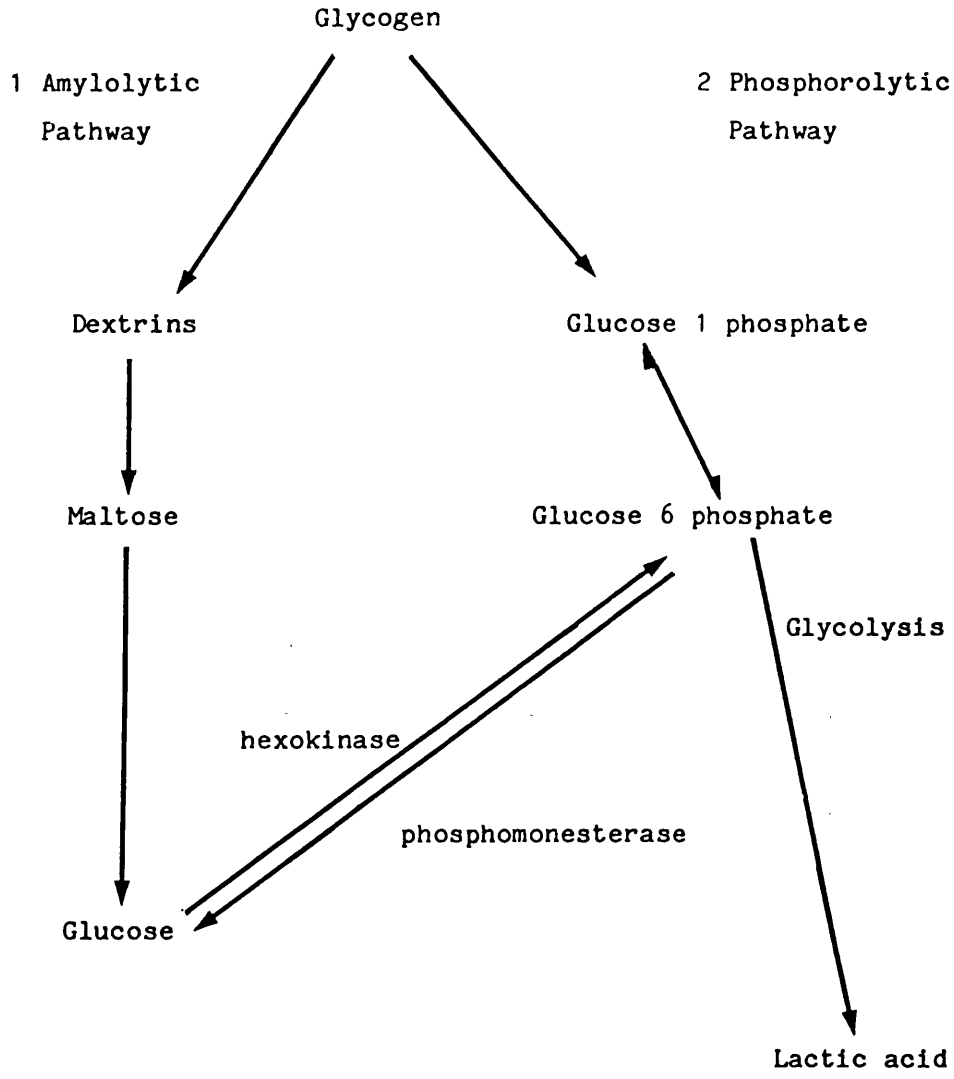
POST-MORTEM CHANGES OCCURRING IN MUSCLE TISSUE*



* Adapted from Gill (1982).

Figure 3

POST MORTEM DEGRADATION OF GLYCOGEN IN FISH AND MEAT *



* Adapted from Eskin et al. (1971)

Thus the ultimate pH is obtained when the process of post mortem glycolysis ceases (Lawrie et al., 1958) and is normally 5.3 to 5.5 (Eskin et al., 1971). Conversely, when little glycogen is available for conversion to lactic acid, as with DFD meat, then a high ultimate pH is obtained (Lawrie, 1985). Glucose concentration declines with an increasing pH, so at a pH of 6.4, as with DFD meat, glucose is absent (Gill, 1982).

3. Microbiology of Stored Red Meat

i. Aerobic Storage at Chill Temperatures

On untreated meat, for example fresh ground beef, there is usually a large population of Gram-negative bacteria (3.5×10^6 g^{-1}) accompanied by a low count (8.7×10^3 g^{-1}) of yeasts (Jay and Margitic, 1981). The relative rates of growth of yeasts vs bacteria probably favour the latter.

Ayres (1960a) recovered species of Candida, Torulopsis and Rhodotorula from refrigerated beef. In addition to these species, Hseih and Jay (1984) found Trichosporon and Cryptococcus spp. but 82% of the yeasts were Candida spp. - C. lipolytica and C. lambica - being predominant on fresh beef whereas C. lipolytica and C. zeylanoides dominated after spoilage. The results of Nychas (1984), who studied the microflora of minced beef, were in agreement with Hseih and Jay (1984) in that he found Candida spp. dominated, and species of Pichia, Cryptococcus, Trichosporon, Rhodotorula and Debaryomyces were present in small numbers only. The yeast flora of fresh pork (Table 9) is composed predominantly of Torulopsis, Candida and Debaryomyces spp. (Szczepaniak et al., 1975).

Only a few of the initial contaminants of meat are able to proliferate under the storage conditions used in the meat industry (Mossel, 1971; McMeekin, 1982). Of the many selective factors influencing microbial colonisation of meat, temperature is the most important (Ayres, 1960a; Nottingham, 1982). Spoilage microorganisms can grow from -5° to +70°C (Mossel and Ingram, 1955) but, with the widespread use of refrigeration at all stages of meat production, those growing at low temperatures (0-7°C) become dominant, due to their rapid growth at these temperatures (Ingram and Dainty, 1971; Gill and Newton, 1977).

Pseudomonads are now recognised as the major spoilage organisms of aerobically stored meat, e.g. beef, at chill temperatures (Gill and Newton, 1977, 1978; Gill, 1980, 1983; McMeekin, 1982; Shaw and Latty, 1982, 1984; Shaw and Dainty, 1985). Earlier studies (Haines, 1933a; Empey and Vickery, 1933; Empey and Scott, 1939), however, identified 90 to 100% of the spoilage microflora with the inadequately defined genus Achromobacter and only 1 to 4% with Pseudomonas spp. Indeed many isolates assigned to Achromobacter (e.g. Empey and Scott, 1939) were subsequently shown to be strains of Pseudomonas (Brown and Weidemann, 1958) or Moraxella/ Acinetobacter (Dainty et al., 1983). These problems with the identification of bacterial isolates from meat led to the view that spoilage was due to organisms of the ill-defined Pseudomonas/ Achromobacter group (Kirsch et al., 1952; Ayres, 1960a; Ingram and Shewan, 1960; Gardner, 1965; Gardner et al., 1967; Ingram and Dainty, 1971).

The taxonomy of the genus Pseudomonas in general was put on a sound basis by the study of Stanier et al. (1966). Subsequent studies showed that the non-fluorescent Ps. fragi dominates the flora of chilled meat (Stringer et al., 1969; Shaw and Latty, 1981, 1982, 1984; Banks and Board, 1983) in association with Ps. lundensis and to a lesser extent Ps. fluorescens particularly strains of certain clusters of Biovar V (Palleroni et al., 1973; Molin and Ternstrom, 1983). Other Gram-negative psychrotrophic bacteria isolated from red meat, Acinetobacter and Moraxella (Ingram and Simonsen, 1980; Gill and Newton, 1982) remain subordinate members of the spoilage flora. DFD meat (see Section 2) stored under aerobic conditions yields a microbial flora similar to that of normal meat, the only distinguishing feature being that Pseudomonas spp. account for 88% of the population on meat at pH 6.6 as opposed to 76% on meat of normal pH (Erichsen and Molin, 1981).

The microbiology of lamb has not been studied as extensively as that of beef. As with chilled beef, Patterson (1970) found that the spoilage of minced lamb at 4°C was due to the Pseudomonas-Achromobacter group. Dainty et al. (1983) confirmed this observation. They found that 42-60% of the microflora were Pseudomonas spp. on beef, pork or lamb stored under a gas-permeable film. In contrast, Barlow and Kitchell (1966) noted that the microflora of lamb chops packed in an oxygen-permeable film at 5°C, consisted mainly of Br. thermosphacta whereas the microflora of beef stored under the same conditions was dominated by Gram-negative bacteria. Dainty et al. (1983) observed that

Br. thermosphacta accounted for 22% of the population on lamb, 26% on pork and only 4% on beef.

Kelly (1978) noted that when lamb was refrigerated (4°C) for 7 d, 48% of the population were Pseudomonas spp., 23% Br. thermosphacta and 22% Moraxella spp.

Storage at cold temperatures (-5°C) is one of the factors that may select a flora dominated by yeasts. Yeasts represent only 0.1% of the initial microflora of lamb loins wrapped in an oxygen-permeable plastic film but reached a maximum density of 10^6 cm^{-2} after 20 weeks storage at -5°C (Lowry, 1984; Lowry and Gill, 1984). Although 10^6 yeasts cm^{-2} is equivalent to the biomass of 10^8 bacteria cm^{-2}, the level required to initiate superficial spoilage in chilled meat (Gill and Newton, 1978), no off odours or flavours were detectable (Winger and Lowry, 1983). Four species of yeasts were present; Cryptococcus laurentii var. laurentii (90% of the yeast flora) Cr. infirmo-minatus, Trichosporon pullulans and Candida zeylanoides (Lowry, 1984; Lowry and Gill, 1984).

ii. Vacuum-Packed Storage

Some beef carcases are cut into 1.8 - 9 kg primal joints and vacuum-packed at the abattoir and stored in plastic bags (Dainty et al., 1983) with low gas permeability. The trapped atmosphere becomes depleted of oxygen (<1% v/v) due to tissue respiration (Ingram and Simonsen, 1980) and enriched with carbon dioxide -> 20% v/v - (Dainty et al., 1983).

The carbon dioxide inhibits the growth of Gram-negative bacteria (Sutherland et al., 1977) particularly Pseudomonas spp.

(Shaw and Nicol, 1969). The mode of action of carbon dioxide remains speculative (Gill, 1986). Susceptible microorganisms exposed to carbon dioxide show an extension in the lag phase (King and Nagel, 1967; Gill and Tan, 1979, 1980). The growth rate and final count of pseudomonads decreases with decreasing film permeability (Newton and Rigg, 1979) as the less permeable the plastic film the more rapidly carbon dioxide accumulates in packs of meat (Shaw and Dainty, 1985). Hence, Pseudomonas spp. have only occasionally been isolated from vacuum-packed beef (Sutherland et al., 1975). It needs to be stressed that yeasts were not sought in these studies.

Due to the synergistic effect of the increased carbon dioxide and low oxygen concentrations (Gill, 1986), the initial aerobic association (Pseudomonas-Acinetobacter-Moraxella) is replaced by a facultative anaerobic one of Br. thermosphacta and Enterobacteriaceae (Ingram and Simonsen, 1980). The growth rate of Br. thermosphacta is unaltered at high concentrations of carbon dioxide under aerobic and anaerobic conditions (Roth and Clark, 1975). It is therefore unaffected by film permeability (Newton and Rigg, 1979).

Due to relatively rapid growth under anaerobic conditions (Gill, 1982), Gram-positive lactic acid bacteria, mainly "atypical" streptobacteria - Lactobacillus divergens and L. carnis - (Shaw and Harding, 1984, 1985), now reclassified as Carnobacterium divergens and Carn. piscicola (Collins et al., 1987), are able to outgrow the other facultative anaerobes. Antimicrobial substances produced by lactobacilli inhibit the growth of would-be competitors such as

Pseudomonas spp. Br. thermosphacta and Enterobacteriaceae(Gill, 1986). Hence lactic acid bacteria predominate and account for 96% of the microflora of vacuum-packed meat (Erichsen and Molin, 1981).

Brochothrix thermosphacta appears to be more common on vacuum-packed lamb (Patterson and Gibbs, 1978; Shaw et al., 1980) than on vacuum-packed beef. Brochothrix thermosphacta does not grow anaerobically on meat of pH $\leq$ 5.8 (Campbell et al., 1979; Grau, 1980) therefore, growth in this instance can be attributed to differences in pH and permeability of packaging material (Campbell et al., 1979). Lactobacillus spp., however, dominate accounting for 73.9 - 98.8% of the microflora of vacuum-packed lamb chops stored in high-oxygen-barrier (HOB) film (Henry et al., 1983).

With vacuum-packed DFD meat the situation is different. The high pH (6.1-6.6) of DFD meat permits organisms that are usually inhibited by the low pH (5.5-6.0) of normal meat to grow. Thus the Gram-negative Shewanella putrefaciens - formerly known as Alteromonas putrefaciens - MacDonell and Colwell, 1985), Yersinia enterocolitica and Serratia liquefaciens - previously classified as Enterobacter liquefaciens - become dominant (Gill and Newton, 1979; Gill, 1980). Lactic acid bacteria have been shown by some to be a major component of the microbial population of vacuum-packed DFD meat (Patterson and Gibbs, 1977; Dainty et al., 1979). Other workers have reported a microflora of lactic acid bacteria (60%) and Br. thermosphacta (40%) as compared to the former accounting for 96% in normal meat (Erichsen and Molin, 1981). Again, these studies did not look for yeasts.

iii. Storage in Modified Gas Atmospheres

Modified gas atmospheres contain carbon dioxide (10-20%) as an antibacterial agent, it being mixed with air, nitrogen or oxygen (Dainty et al., 1983). Haines (1933b) noted that 10% (v/v) carbon dioxide in air extended the lag phase and doubled the generation time of Pseudomonas spp. Since then it has been found that the growth rate of Pseudomonas spp. decreases as the carbon dioxide concentration increases up to 20% when the growth rate of pseudomonads is reduced by 30% (Gill, 1986). The extent of carbon dioxide inhibition of microbial growth is accentuated by chill temperatures (Gill and Tan, 1979, 1980; Gill, 1986).

Facultative anaerobes, such as Enterobacter spp. and Br. thermosphacta are unaffected by these levels of carbon dioxide whether grown aerobically or anaerobically (Roth and Clark, 1975; Newton et al., 1977; Gill and Tan, 1980). Lactobacilli are able to multiply in 100% carbon dioxide (Roth and Clark, 1975; Sutherland et al., 1977; Enfors and Molin, 1980).

When oxygen is excluded, as with 20% carbon dioxide and 80% nitrogen, the strict aerobic Pseudomonas and Acinetobacter/ Moraxella spp. do not grow (Clark and Burki, 1972; Nottingham, 1982; Gill, 1986). The anaerobic growth of Br. thermosphacta (Grau, 1980) and Shew. putrefaciens is inhibited if the pH of the meat is below 5.8 (Gill, 1986). Enterobacteriaceae are also inhibited under these stricter anaerobic conditions (Grau, 1981). As the lactobacilli grow rapidly at temperatures approaching 0°C, however, they outgrow psychrotrophic Enterobacteriaceae (Newton and Gill, 1978a).

Newton et al. (1977) noted that Br. thermosphacta was a major part of the microflora of lamb chops under all gas mixtures tested and dominated in 20% carbon dioxide with oxygen. Pseudomonads were important in oxygen-containing atmospheres, and Enterobacteriaceae in low oxygen and oxygen-free ones (Newton et al., 1977). Lactobacillus spp. were detected in oxygen-free atmospheres where the growth of the competing organisms was reduced (Newton et al., 1977).

DFD meat stored under a gas mixture 78% nitrogen, 20% carbon dioxide and 2% oxygen had a completely different microflora to that of normal meat stored under the same conditions (Erichsen and Molin, 1981). The microbial population of normal meat consisted of 52% lactic acid bacteria, 16% Enterobacteriaceae and 16% coryneforms, whereas the microflora of DFD meat comprised 44% pseudomonads, 28% lactic acid bacteria and 28% Br. thermosphacta (Erichsen and Molin, 1981). Again, yeasts were not investigated.

4. Chemistry of Spoilage

i. Aerobic Storage

A comprehensive understanding of the chemical changes occurring in meat due to microbial growth has only emerged recently. As the spoilage flora grow on the surface of aerobically stored meat at chill tempertures (Ingram and Dainty, 1971), the biochemical modifications are a surface phenomenon and spatially restricted in terms of the bulk of the meat.

Microbial growth occurs initially at the expense of low molecular weight compounds (Gill, 1986). Bacteria such as strains

of Pseudomonas, Enterobacter and Br. thermosphacta grow aerobically on the meat surface with the preferential utilisation of glucose - Table 12 - (Gill and Newton, 1977). Growth is inhibited if the rate of diffusion of glucose from within the meat is slower than the rate of bacterial uptake (Gill, 1976; Gill and Newton, 1978; Dainty et al., 1983). Enterobacter spp. have a greater affinity for glucose than does Br. thermosphacta, and are therefore able to reduce the glucose concentration on the meat surface and thus prevent the growth of the latter (Newton and Gill, 1978b). Enterobacter spp. have the added advantage also of being able to utilise glucose-6-phosphate - Table 12 - (Newton and Gill, 1978b; Gill and Newton, 1979).

Pseudomonads rapidly convert glucose to gluconate and/or 2-ketogluconate by an extracellular pathway (Eisenberg et al., 1974; Whiting et al., 1976; Farber and Idziak, 1982) thus producing products that are not widely utilised by would-be competitors (Whiting et al., 1976). Gluconate formation may play an important role in reducing glucose to growth limiting levels (Whiting et al., 1976). Under glucose limitation the transport of gluconate is inhibited and glucose uptake is increased (Whiting et al., 1976). Thus the extracellular oxidation route is replaced by an intracellular phosphorylative pathway (Whiting et al., 1976).

While glucose is available the enzymes required for metabolising other substrates, such as amino acids, are subject to catabolite repression and inhibition (Jacoby, 1964; Ornoston, 1971). When the bacterial density reaches 10^8 cells cm^{-2}, the glucose concentration at the meat surface is zero (Gill, 1976). The

Table 12. Order of utilisation of individual low molecular weight components of meat for bacterial growth*

Substrate	Aerobic					Anaerobic		
	Non-fluorescent Pseudomonas	Fluorescent Pseudomonas	Acinetobacter	Enterobacter	Brochothrix thermosphacta	Lactobaccillus	Enterobacter	Brochothrix thermosphacta
Glucose	1	1	-	1	1	1	1	1
Glucose-6-phosphate	-	-	-	2	-	-	2	-
Glycogen	-	-	-	-	-	NL	NL	NL
Amino acids	2	2	1	3	2 (glutamate only)	2 (arginine only)	-	-
Lactate	3	3	2	4	-	-	-	-

- not utilized

NL not in literature

*Order of utilization adapted from Nottingham et al. (1981); Gill (1982, 1986) based on data from Gill and Newton (1977).

Glycogen data from Gill and Newton (1977).

resultant breakdown of amino acids produces "off-odours" consisting of malodorous sulphides, esters and amines (Dainty et al., 1983, 1984; Edwards et al., 1983) with a concomitant increase in pH associated with ammonia production (Jay and Kontou, 1967). Hence the glucose concentration in meat directly determines the cell density achieved before spoilage is manifested (Gill, 1986). Conversely, when 2% glucose (by weight) was added to ground beef of normal pH, the growth of pseudomonads was inhibited by the accumulation of acid products thus delaying the occurrence of slime and "off-odours" and thereby prolonging shelf-life (Shelef, 1977).

Acinetobacter spp. are unable to metabolise hexoses (Gill and Newton, 1978), and utilise amino acids (Table 12) as the primary growth substrate (Gill and Newton, 1977). Pseudomonas spp. still grow at maximum rate by degrading amino acids as the secondary substrate (Gill and Newton, 1977, 1978; Gill, 1982). Lactic acid is an important alternative substrate because its concentration increases post rigor (Table 11). Moreover, the sparing action of glucose means that secondary substrates such as amino acids and lactic acid do not become depleted at the meat surface (Gill, 1976). In fact, amino acids are still abundant at the surface when aerobic bacterial growth ceases due to oxygen limitation (Gill and Newton, 1978).

The degradation of low molecular weight compounds such as free amino acids and nucleotides has a sparing action as they are utilised in preference to complex lipids and proteins (Jay, 1972; Jay and Shelef, 1976; Gill and Newton, 1980). At the time of advanced spoilage, soluble sarcoplasmic proteins are degraded (Jay,

1966) before complex myofibrillar proteins are utilised by bacteria such as Ps. fragi (Borton et al., 1970a, b; Hasegawa et al., 1970a, b; Tarrant et al., 1971; Dainty et al., 1975). Although lipid breakdown does not seem to be necessary for spoilage of adipose tissue (Gill and Newton, 1980), bacteria such as lactobacilli and Br. thermosphacta, have the ability to produce lipases particularly when the glucose concentration is low (Papon and Talon, 1988). Yeasts on the other hand are considered to be the major cause of beef fat lipolysis (Lea, 1931; Ingram, 1958) and their lipolytic activity may enable them to compete successfully in the microflora selected by fatty parts of meat (Aboukheir and Kilbertus, 1974). Indeed, Candida, Cryptococcus and Trichosporon spp. have been noted to produce lipases (Johannsen et al., 1984).

When pseudomonads are predominant in the flora, spoilage odours occur with amino acid breakdown (Gill, 1976). When Br. thermosphacta was a major component of the flora, however, spoilage coincided with carbohydrate breakdown (Dainty and Hibbard, 1983) and the resulting sweet "off-odours" were due to metabolic products such as acetoin, acetic acid, isobutyric, isovaleric and 2-methyl butyric acids (Dainty and Hibbard, 1980, 1983).

Spoilage follows a different pathway in DFD meat. Since glucose and glucose-6-phosphate occur in very low concentrations if at all, amino acids are immediately attacked by bacteria (Newton and Gill, 1980; Gill, 1986). Thus spoilage occurs at lower microbial numbers (10^6 cm^{-2}) and hence more rapidly than in meat of normal pH (Newton and Gill, 1978a, 1980; Gill, 1986). Indeed, Newton and Gill (1978a) found that the addition of glucose (100 μg g^{-1} wet wt) to DFD meat

extended the shelf-life from 2 d (with no glucose) to 4 d (with glucose). Glucose may be added routinely to carcasses or meat to prevent early spoilage of undetected DFD meat (Gill, 1986).

ii. Vacuum-Packed Storage

In anaerobic conditions glucose is the preferred fermentative substrate for Br. thermosphacta, Enterobacter spp. and lactobacilli (Newton and Gill, 1978a). Although lactobacilli have a lower affinity for glucose than Enterobacter spp. or Br. thermosphacta and are unable to utilise glucose-6-phosphate, they dominate in vacuum-packed meat through the production of antimicrobial substances (Newton and Gill, 1978b). Although lactic acid and acetic acid, hydrogen peroxide and antibiotics have been listed in the literature (Wilkins and Board, 1989) as possible causal agents, the identity of the antibacterial compound has yet to be established. Lactic acid has been dismissed as a potential antimicrobial agent simply because it is also produced by Br. thermosphacta (McLean and Sulzbacher, 1953) without evident interference of the growth of other organisms. The most probable agent may be an antibiotic polypeptide analogous to "Nisin" (Mattick and Hirsch, 1947; Hurst, 1981) or a similar bacteriocin reported to be produced by lactic acid bacteria (Klaenhammer, 1988). Recently it has been found that Ps. fragi was inhibited by a small (mol. wt $\geq$700) compound with an aromatic moiety produced by Lactobacillus bulgaricus during growth in nutrient broth (Abdel-Bar et al., 1987).

The lactobacilli ferment glucose to lactate, acetate and other short chain fatty acids that impart a sour, acid "cheesy" odour to vacuum-packed meat (Dainty et al., 1979; Shaw and Dainty, 1985). When glucose is exhausted lactobacilli can metabolise arginine (Rogosa and Sharpe, 1960; Gill, 1976; Newton and Gill, 1978b). One mol of glucose yields 3 ATP but only 1 ATP is produced per mol of arginine (Thornley, 1960), therefore the fermentation of the latter does not lead to an increase in numbers of lactobacilli (Gill, 1976) and thus cell density will still depend on the rate of diffusion of glucose from the underlying meat (Gill, 1976). The resultant catabolism of amino acids, however, produces off-odours associated with amines such as cadaverine, putrescine and tyramine (Edwards et al., 1985, 1987).

When glucose is added to vacuum-packed meat of normal pH (5.8-6.8) there is a resultant increase in the final numbers of lactobacilli and the accumulation of volatile acids is accentuated (Newton and Gill, 1980). Glucose, however, can be added to vacuum-packed meat on opening for display purposes as it replaces the glucose exhausted by the anaerobic flora and delays amino acid breakdown by pseudomonads, and therefore the onset of spoilage (Gill, 1986).

High pH, oxygen limitation and the absence of glucose in vacuum-packed DFD meat enables Serratia liquefaciens and Shew. putrefaciens to compete successfully with lactobacilli (Gill and Newton, 1979). Serratia liquefaciens utilises serine in conjunction with glucose and glucose-6-phosphate in meat of normal pH (Newton and Gill, 1978b; Gill and Newton, 1979). Shewanella putrefaciens

utilises serine preferentially or at the same time as glucose (Gill and Newton, 1979). In the absence of glucose, Ser. liquefaciens and Shew. putrefaciens breakdown amino acids - lysine, arginine, threonine (Gill and Newton, 1979).

The offensive putrid smells of vacuum-packed DFD meat (Patterson and Gibbs, 1977) are caused by Ser. liquefaciens breaking down amino acids to sulphides and amines (Gill and Newton, 1979; Dainty et al., 1983). Shewanella putrefaciens produces hydrogen sulphide from cysteine (Gill and Newton, 1977) which converts the muscle pigment, myoglobin, to green sulphmyoglobin (Nicol et al., 1970) causing a condition known as "greening" (Taylor and Shaw, 1977).

The addition of glucose or citrate to vacuum-packed DFD meat prevents "off-odours" by Ser. liquefaciens as the addition of the preferred substrate delays amino-acid breakdown (Newton and Gill, 1980). Addition of glucose at a high pH, however, favours the growth of Br. thermosphacta and stimulates the growth of Shew. putrefaciens (Gill, 1986). Greening is not prevented as Shew.putrefaciens degrades cysteine in the presence of glucose (Newton and Gill, 1980). Growth of Shew. putrefaciens, however, can be inhibited by lowering the pH to <6.0 (Gill and Newton, 1979) with citrate buffer (Newton and Gill, 1980). Anaerobic growth of Br. thermosphacta is also inhibited at pH <6.0 (Campbell et al., 1979; Grau, 1980).

This review has emphasised that the most commonly used processing and storage methods for red meats appears to select a bacterial flora even though yeasts are initially important

contaminants. With an a_w above 0.94 and temperature of 6-7°C an interplay of pH, pO_2, pCO_2 and glucose selects a particular combination of bacterial species. The study by Lowry (1984) of lamb stored at -5°C provides an exception in that yeasts were selected during 20 weeks of storage. In this instance it is probable that a lowered a_w as well as temperature were the elective factors. Indeed, the selection of yeasts by low a_w and/or low pH is a feature of processed meats. Thus Candida, Debaryomyces and Torulopsis spp. (Table 9) are present in cured fermented sausage, country cured ham (Leistner and Bem, 1970) salami, dry sausage (Comi and Cantoni, 1980a,b) and bacon (Gardner, 1983). Smith and Hadlok (1976) isolated Deb. hansenii and T. candida from cured raw meat products. Debaryomyces membranaefaciens var. hollandicus and Deb. kloeckeri were commonly associated with meat brines (Costilow et al., 1954).

When minced beef is γ irradiated the bacteria are inhibited whereas the number of psychrotrophic yeasts increases (Johannsen et al., 1984). The yeast population on untreated crab meat is low (1×10^3 g^{-1}) but, again following irradiation, the yeast flora exceeded 1×10^5 g^{-1} with an extended shelf life (Eklund et al., 1966). The yeast genera recovered from untreated or irradiated crab meat were Rhodotorula, Cryptococcus, Torulopsis, Candida and Trichosporon (Eklund et al., 1965, 1966). When frankfurters were irradiated the surface flora consisted mainly of Deb. subglobosus and T. candida (Drake et al., 1959).

Antibiotic treatment of meat inhibits bacteria and hence favours the growth of yeasts. This was noted when poultry meat was

treated with chlortetracycline, oxytetracycline or tetracycline (Ayres et al., 1956; Njoku-Obi et al., 1957; Wells and Stadelman, 1958; Walker and Ayres, 1959). The yeast population increased from 10^4-10^5 cm^{-2} on untreated to 10^5-10^8 cm^{-2} with poultry treated with 10 ppm oxytetracycline (Wells and Stadelman, 1958). The yeast flora was dominated by species of Rhodotorula (80%), Torulopsis and Cryptococcus (Wells and Stadelman, 1958). Torulopsis, Geotrichum and Rhodotorula spp. dominated the yeast flora on untreated poultry whereas Saccharomyces spp. dominated on chlortetracycline treated chicken meat (Njoku-Obi et al., 1957).

The growth of the dominant meat spoilage bacteria, such as pseudomonads, is inhibited by the addition of sulphite at the permitted level of 450 µg SO_2 g^{-1} (Anon., 1974) to British fresh sausages (Banks and Board, 1981). The reduction of competition (Dalton et al., 1984) and the fact that yeasts are tolerant of the preservative (Brown, 1977; Banks, 1983) permits the establishment of a yeast flora. The sulphite present in sausages did not affect the numbers and range of yeasts. It did, however, favour the growth of Deb. hansenii and Candida spp. thereby reducing the proportion of Cryptococcus and Rhodotorula spp. (Dalton et al., 1984).

The yeasts occurring on sausages have been under investigation since Kühl (1910) isolated such organisms from the surface slime of dried sausages and Cary (1916) observed the presence of yeasts on sausages. In 1920 Cesari and Guilliermond described unnamed yeasts from slimy sausage that were later identified with Deb. hansenii and Deb. kloeckeri by Lodder and Kregen-van Rij (1952) the latter of which was reclassified as Deb. hansenii (Lodder, 1970). Mrak and

Bonar (1938) isolated Deb. guilliermondii var. nova zeelandicus from slimy Wiener sausages. This organism was identified with Deb. hansenii by Lodder and Kreger-van Rij (1952).

Yeasts are common contaminants of British fresh sausage (Dowdell and Board, 1968). As the size of the yeast population ranges from $1 \times 10^{1} - 2.4 \times 10^{4}\ g^{-1}$ of product it is reasonable to assume that they may play a significant role in spoilage. Skins of stale sausages were found to be covered in a thick yellow green film of yeast (Dowdell and Board, 1971). Abbiss (1978) noted that T. candida was the major species on sausages dominated by yeasts whereas Tr. cutaneum was the main contaminant on sausages dominated by Br. thermosphacta. Banks (1983) observed that Candida or Torulopsis spp. were the main yeasts on sausages. Živanović and Ristić (1974) had also found that Candida spp. dominated the tea sausages. Candida iberica was first isolated from "salchichón" (Spanish sausage) by Ramírez and González (1972). Candida melini and Rh. mucilaginosa were the most frequent species isolated from sausages (Table 9) by Aboukheir and Kilbertus (1974). Pathogenic Candida spp., such as C. parapsilosis and C. tropicalis, have been recovered from Bologna type sausage, salami sausage and ham (Staib et al., 1980), whereas Dalton (1984) and Dalton et al. (1984) found that the most common species on unsulphited and sulphited sausages and minced beef were Deb. hansenii followed by C. zeylanoides and Pichia membranaefaciens. Nitrite assimilating species of Debaryomyces had been isolated from luncheon meat by Wickerham (1957).

5. Sulphite as a Food Preservative

Six sulphiting agents - sulphur dioxide, sodium sulphite, sodium and potassium bisulphite, sodium and potassium metabisulphite are commonly used in beverages with an acid pH (wines and ciders) and foods with a neutral pH (meat products). Sulphite maintains the fresh colour of meat (Leads, 1979; Sullivan and Smith, 1985) by preventing the formation of metmyoglobin from oxymyoglobin (Roberts and McWeeny, 1972). Its main use, however, is as an antimicrobial agent. In wines and ciders, for example, sulphite inhibits the growth of wild yeasts and bacteria (Cruess, 1912; Ough and Crowell, 1987). In meat and meat products, the growth of the main spoilage bacteria, the Gram-negative pseudomonads, is suppressed by sulphite (Richardson, 1970; Banks et al., 1985c) permitting the development of a yeast flora associated with acetaldehyde production and sulphite binding, analogous to that in fermented wines (Dalton, 1984).

Effect of pH on the antimicrobial activity of sulphite

It is well established that the degree of ionisation determines the efficacy of sulphite as an antimicrobial agent (Douglas, 1966; Hammond and Carr, 1976). Sulphur dioxide dissolved in water forms sulphurous acid (H_2SO_3) which dissociates into molecular sulphur dioxide (SO_2), bisulphite ions (HSO_3^-) and sulphite ions (SO_3^{2-}) in a pH dependent equilibria (King et al., 1981). Sulphurous acid is dibasic and has two dissociation constants (Rahn and Conn, 1944).

$$\frac{[H][HSO_3]}{[H_2SO_3]} = k_1$$

$$\frac{[H][SO_3]}{[HSO_3]} = k_2$$

Rahn and Conn (1944) calculated the first dissociation constant, k_1, to be 1.7×10^{-2}. Vas and Ingram (1949) and King et al. (1981) accepted this value. The second dissociation constant, k_2, published by Rahn and Conn (1944) of 5×10^{-6} was repeatedly quoted in the literature viz Vas and Ingram (1949), Joslyn and Braverman (1954) and Rehm and Wittmann (1962). Subsequent investigations, (King et al., 1981) revealed that it was in error by two pH units and a new value 6.31×10^{-8}, for k_2 was proposed. Even with these new values (pk_1 = pH 1.77 and pk_2 = pH 7.20), the percentage of molecular sulphur dioxide present at the pH of meat products (5.8-6.8) is negligible (King et al., 1981).

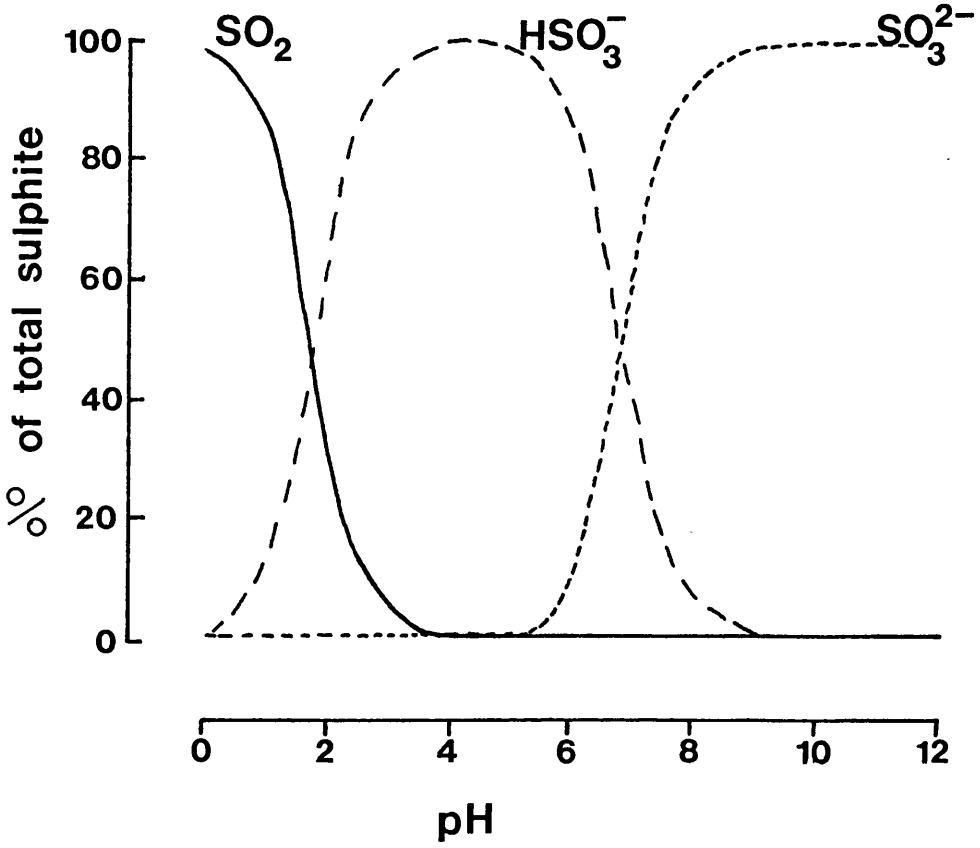
Molecular sulphur dioxide is the most effective antimicrobial agent with bisulphite being more active than sulphite ions (Rehm and Wittmann, 1962, 1963; Babich and Stotzky, 1978). With reference to King et al. (1981), the percentage of molecular sulphur dioxide is higher at a low pH (Fig. 4). The preservative is therefore more effective in acid products such as cider (Burroughs, 1964; Burroughs and Sparks, 1964), orange juice (Ingram and Vas, 1950a, b) and wines (Weeks, 1969; Burroughs, 1974, 1981). In contrast to the acid pH of 2.8-4.2 found in wines (Ough and Crowell, 1987), meat products have a pH of 5.8-6.8. At this high pH the only antimicrobial components are the bisulphite and sulphite ions (Hammond and Carr, 1976).

The fate of sulphite during processing and storage of a product

Only the free sulphite is antimicrobial (Burroughs and Sparks, 1964). It is important, therefore, to monitor the percentage of

Figure 4

THE DISTRIBUTION OF MOLECULAR SULPHUR DIOXIDE, BISULPHITE AND SULPHITE IONS IN AQUEOUS SOLUTION *



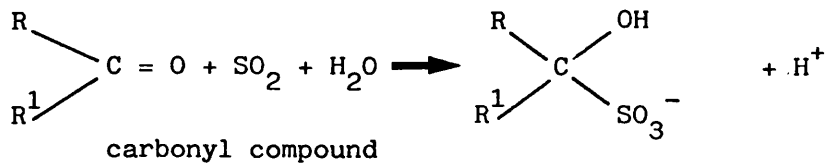
* Adapted from King et al. (1981)

free sulphite lost during processing and storage of a product. In the United Kingdom sulphite is added to uncooked, comminuted meat products e.g. sausages, which contain cereal (Kidney, 1974; Wedzicha, 1984) to give a final concentration of 450 μg SO_2 g^{-1} (Anon., 1974). To achieve this level, initially 600 μg g^{-1} sulphite (Banks and Board, 1982b) is added which immediately falls by ca 20-30% during the mixing of the sausage ingredients (Anderton and Locke, 1956; Banks, 1983; Dalton, 1984) and it continues to diminish with storage (Kidney, 1974; Brown, 1977; Legan, 1981; Banks, 1983; Dalton, 1984). It has been surmised that some of the sulphite is irreversibly lost by enzymic or non-enzymic autooxidation to sulphate (Brown, 1977; Wedzicha, 1987). This occurs via a catalyst-sensitive chain reaction (Abel, 1913; Schroeter, 1966) or free radical intermediates (Fridovich and Handler, 1961). These free radicals are scavenged and cause autooxidation of unsaturated organic compounds such as fats, in which sulphur is readily incorporated (Wedzicha, 1987). A large proportion, however, of the bisulphite and sulphite ions are reversibly bound to compounds of meat or microbial origin (Brown, 1977) thus reducing the preservative concentration and hence its antimicrobial activity (Neuberg, 1929; Ingram, 1949; Rehm, 1964).

Our understanding of sulphite binding in meat products, such as sausages, was helped by a modification of the steam-distillation technique. With the original technique (Monier-Williams, 1927) the sample was acidified with orthophosphoric acid and boiled under reflux for 60 min. Sulphur dioxide carried over in inert gas was collected in hydrogen peroxide and then titrated with sodium

hydroxide. The modification allowed the sample to be stirred for 15 min in hydrochloric acid to collect free sulphur dioxide and then heated for 15 min to recover bound sulphur dioxide in 5-5'-dithiobis 2-nitrobenzoic acid (DTNB). The colour change associated with collection of sulphur dioxide is analysed by spectrophotometry (Banks and Board, 1982a; Banks, 1983). In this way the percentages of free, bound and total sulphite could be calculated over the storage time of a product (Banks and Board, 1982a; Banks, 1983). Banks (1983) estimated that 26% of the sulphite was lost irreversibly and a further 23% was bound during the manufacturing of sausages. On storage free sulphite was lost more rapidly than total sulphite. The rate of sulphite loss at 15°C and 22°C was greater than at chill temperatures (Abbiss, 1978; Banks, 1983; Dalton, 1984). Thus a higher percentage of bound sulphite was present at 15°C and 22°C than at 1°, 4° or 5°C (Banks, 1983; Dalton, 1984).

Sulphite is a highly reactive molecule (Joslyn and Braverman, 1954; Carr et al., 1976) which combines readily with constituents of foods and beverages (Schroeter, 1966; Burroughs and Sparks, 1964, 1973a). It binds reversibly with substances containing one or two carbonyl groups (aldehyde or keto groups) to form carbonyl bisulphite compounds - "hydroxysulphonates" - (Burroughs, 1964; Burroughs and Sparks, 1964, 1973a, b, c; Hammond and Carr, 1976).



carbonyl compound

Hydroxysulphonate

(Hammond and Carr, 1976)

Sulphite also forms hydroxysulphonates with sugars (Ingram, 1948) and amine bisulphites with amines (Joslyn and Braverman, 1954). An equilibrium is established between the free and bound sulphite so that, if more sulphur dioxide is added, bound sulphite increases and conversely if sulphur dioxide is removed, sulphite is released from the carbonyl bisulphite compounds (Burroughs, 1964; Burroughs and Sparks, 1973b).

The equilibrium set up is defined as:

$$k = \frac{[S][X - x]}{[x]}$$

where k is the equilibrium constant, [S] the molecular concentration of free sulphite in any form, [X] the total molecular concentration of carbonyl compounds (free and bound) and [x] the molecular concentration of the undissociated bisulphite compound (Burroughs, 1964; Burroughs and Sparks, 1973a,b). The position of the equilibrium depends on pH, temperature and the concentration of the free sulphite (Burroughs and Sparks, 1973b,c) and is independent of the total carbonyl concentration.

Between pH 1 to 7, acetaldehyde binds sulphite almost completely (Rankine and Pocock, 1969). Consequently it will bind a higher percentage of available sulphite than substances having a lower binding potential. All aldehydes rapidly form hydroxysulphonates with bisulphites. Ketones react only slowly (Joslyn and Braverman, 1954) and sugars, such as glucose and arabinose, at a slower rate still (Joslyn and Braverman, 1954). Glucose-sulphonate is unstable and dissociates completely at pH 6

and 7 (Braverman, 1953). If acetaldehyde is added to a solution containing glucose bisulphite, it replaces glucose in combination thereby forming the more stable compound, acetaldehyde-bisulphite (Joslyn and Braverman, 1954).

Several potential binding compounds, many probably of microbial origin, have been isolated from wines and ciders viz D-xylose, L-xylosone, acetaldehyde, D-galacturonic acid, D-threo-2,5-hexodiulose, 2,5-diketogluconic acid, 2-ketogluconic acid, α-ketoglutaric acid and pyruvic acid (Burroughs, 1964; Burroughs and Sparks, 1973a,b). Sugars which are known to have little binding power were implicated originally in sulphur dioxide binding in wine (Farnsteiner, 1904). Kerp (1904) suggested, on the other hand, that acetaldehyde was an important binding agent. Subsequently acetaldehyde, pyruvic and α-ketoglutaric acids have emerged as the three most important binding agents in white wines (Rankine and Pocock, 1969; Weeks, 1969), these accounted for almost all of the bound sulphite.

During storage, the concentration of the potential sulphite binding compounds increases in cider (Burroughs and Sparks, 1973c) and in sausages (Banks, 1983; Dalton, 1984). It needs to be stressed that as sulphite induces the production, these compounds are presumably of microbial origin (Burroughs and Sparks, 1973c). Brown (1977) noted that sulphite binding was evident around microbial colonies in sausage slurries. Dalton (1984) found a positive correlation between the yeast population size and the extent of sulphite bound in minced pork ($r = 0.98$ at 1°C, $r = 0.92$ at 15°C storage).

Banks (1983) observed an extensive loss of free sulphite from sausages dominated by yeasts. He concluded that these organisms were producing binding compounds, which Brown (1977) assumed would be mainly acetaldehyde. Sausages contain glucose and maltose (Abbiss, 1978), substrates that can be metabolised by yeasts to acetaldehyde, pyruvate and α-ketoglutarate. These three potential binding compounds are known to be produced by yeasts (Rankine and Pocock, 1969). Dalton (1984) noted that the acetaldehyde concentration in sausages was positively related to the amount of sulphite bound ($r = 0.89$, $p = <0.05$). She found also that yeasts were the only members of the microbial association of sausages to produce acetaldehyde in the presence of sulphite.

Further evidence for sulphite induction of acetaldehyde secretion was presented by Stratford (1983). Saccharomycodes ludwigii was found to secrete acetaldehyde only when inoculated into a sulphited broth. Secretion ceased when all the free sulphite was bound. As the acetaldehyde concentration was too high to be accounted for by cell storage, he concluded that acetaldehyde production was induced by sulphite. Indeed, Neuberg and Reinfurth (1918, 1919) had found that the amount of acetaldehyde produced increased with the amount of sulphite added in alcoholic fermentations.

The selective action of sulphite

Sulphur dioxide inhibits the growth of "wild yeasts", moulds and vinegar bacteria thereby selecting fermentative yeasts (Cruess, 1912). Lactic acid bacteria, also, are inhibited by sulphur dioxide

in acidic products such as wine or cider (Fornachon, 1963; Lafon-Lafourcade and Peynaud, 1974; Carr et al., 1976). The proportion of ionic species, SO_2, HSO_3^-, SO_3^{2-}, however, differ markedly with change in pH, therefore bisulphite and sulphite ions rather than molecular sulphur dioxide are present in meats and meat products. It is for this reason that the following review concentrates on these two moieties.

Lafontaine et al. (1955) and Christian (1963) noted that the inhibition of the microflora on raw minced meat was influenced by the concentration of sulphite, the storage temperature and the initial level of contamination. A concentration of 300 $\mu g\ g^{-1}$ sulphite was bacteriostatic at 4°C whereas a higher concentration (1000 $\mu g\ SO_2\ g^{-1}$) was apparently required at 22°C (Lafontaine et al., 1955). Krol and Moerman (1959/1960) also found that 300 $\mu g\ g^{-1}$ sulphite inhibited the microflora of minced meat balls at refrigeration temperatures; a concentration of 900 $\mu g\ SO_2\ g^{-1}$ killed those microorganisms which were likely to be recovered by their isolation methods. As recent work (Banks, 1983; Dalton, 1984) has shown that sulphite is bound more rapidly at higher temperatures, the apparent lowered antimicrobial activity of sulphite at 22°C may reflect merely the temperature-induced differences in binding.

Gram-negative bacteria appear to be the most susceptible to sulphite inhibition in neutral pH products (Christian, 1963; Dyett and Shelley, 1962, 1966; Gardner, 1965, 1968; Richardson, 1970). In unsulphited meat products Ps. fragi dominates at chill temperatures and Enterobacteriaceae dominate at ambient temperatures (Banks

et al., 1985c). The addition of sulphite, however, selects Gram-positive bacteria that produce less offensive souring-odours (Christian, 1963; Gardner, 1965, 1968). Krol and Moerman (1959/1960) found that Enterobacteriaceae were killed with 300 $\mu g\ g^{-1}$ sulphite in minced meat balls stored for 6 d at refrigeration temperatures. Fournaud et al. (1971) also observed that Enterobacteriaceae were particularly sensitive to sulphite in minced pork and saucissons. Dyett and Shelley (1962, 1966) noted that sulphite inhibited coliforms, particularly Salmonella spp., in beef or pork sausages. When the storage temperature was below 22°C, "coli-aerogenes" and other Gram-negative bacteria were suppressed (Dyett and Shelley, 1962, 1966). These observations are in accord with those of Banks and Board (1982b) who found that sulphite prevented the growth of Enterobacteriaceae at 4°C, 10° and 15°C but not at 22°C. They also observed that sulphite had a selective affect on this very large family of fermentative organisms. Thus in untreated sausages Enterobacter agglomerans and two biotypes of Hafnia alvei dominated the Enterobacteriaceae flora, whereas Enterobacter cloacae and Escherichia coli proliferated in sulphited ones (Banks and Board, 1982b; Banks et al., 1987).

Christian (1963) noted that the addition of 3.5 grains of sulphite per lb of minced beef extended the shelf life 2 to 3 fold at chill temperatures. Gardner (1968) also observed a similar response with sulphited vacuum-packed baconburgers; shelf-life was extended by 2 d at 22°C, 10 at 10°C and 28 at 5°C. The composition of the microbial association was not greatly affected. This may have been due, in part, to the nitrite and nitrate influencing the

antimicrobial effect of sulphite (Tompkin et al., 1980). Both Dyett and Shelley (1966) and Gardner (1968) observed that initially sulphite exerted a lethal affect on the microflora and that subsequent growth throughout storage was retarded vis à vis that in control samples. The initial effect may well be due to the larger proportion of free sulphite at the commencement of storage (Brown, 1977; Banks, 1983; Dalton, 1984).

Sulphite increases the lag phase and suppresses the rate of growth of the dominant organisms at refrigeration temperatures (Christian, 1963). It is tempting to speculate that the sulphite inhibition of the growth of the Gram-negative bacteria removes the competition that would otherwise prevent the growth of the much slower growing Gram-positive bacteria. Indeed, this view is supported by the results obtained in an extensive study of the microflora of the sulphited British fresh sausage (Dowdell and Board, 1967, 1968, 1971). The "microbial association" in these studies was dominated by Br. thermosphacta, yeasts and homofermentative Lactobacillus spp. Association of similar composition was noted by Hurst (1972), Ashworth et al. (1974), Tyson (1976), Brown (1977), Abbiss (1978), Leads (1979), Banks (1983) and Banks et al., (1985c) in their studies of sulphited meat and meat products.

Hurst (1972) proposed that sulphite elected a restricted group of microorganisms. Her contention was supported by Brown (1977) and Banks and Board (1981). The growth of the major contaminants of sausages is affected by sulphite at the permitted level of 450 μg SO_2 g^{-1} (Brown, 1977), suggesting that sulphite-tolerant

microorganisms have been elected. Tyson (1976) noted that the addition of polyphosphates to sausage meat enhanced the antimicrobial activity of sulphite. She also found that, once the free sulphite concentration had fallen below a critical level, pseudomonads were able to grow. Similar results were obtained with Enterobacteriaceae (Banks and Board, 1982b; Banks et al., 1987). Inhibition of Enterobacteriaceae was released when the sulphite level dropped to below 50 μg SO_2 g^{-1} sausage or when sulphite was deliberately bound to acetaldehyde or neutralised by hydrogen peroxide (Banks and Board, 1982b; Banks et al., 1987). In contrast Salmonella spp. were not released from growth stasis even after the antimicrobial affect of sulphite had been nullified (Banks and Board, 1982b; Banks et al., 1987).

Banks (1983) proposed that the contribution of particular microorganisms to the microbial association of sulphited meat products, such as sausages, reflects their tolerance of free sulphite in broth cultures. Dowdell and Board (1971) showed that the dominant organisms in sausages Br. thermosphacta, yeasts and homofermentative Lactobacillus spp. - grew in sulphited media whereas the minor members, pseudomonads and coliforms, did not. Banks (1983) found that yeasts (species of Candida, Cryptococcus and Rhodotorula) grew in broth poised at pH 7.0 with a sulphite concentration of 750 μg SO_2 ml^{-1}. Some strains of Br. thermosphacta were inhibited at 450 μg SO_2 ml^{-1} whereas others were tolerant of 750 μg SO_2 ml^{-1}. The growth of Streptococcus spp. was inhibited at μg ml^{-1} sulphite concentration of 310-410, Lactobacillus spp. at 250-400, Pseudomonas spp. at 160-330 and Enterobacteriaceae spp. at

50-270 μg SO_2 ml^{-1}. Salmonella spp. were inhibited by exceptionally low concentrations, 15-109 μg SO_2 ml^{-1} (Banks, 1983).

Yeasts had the highest resistance to sulphite (Banks, 1983). Sulphite tolerance, however, does not develop due to adaptation (Cruess, 1912; King et al., 1981). The literature on wines and ciders demonstrates clearly that fermentative yeasts are more tolerant of sulphur dioxide than are ones with oxidative metabolisms (Rehm and Wittmann, 1962; Reed and Peppler, 1973; Beech and Thomas, 1985). Species of Saccharomyces and Zygosaccharomyces are more tolerant than those of Hansenula, Pichia, Torulopsis, Candida (Rehm and Wittman, 1962), Kloeckera and Rhodotorula (Goto, 1980). Reed and Peppler (1973) observed that Kloeckera apiculata and Pichia membranaefaciens were sensitive to sulphur dioxide whereas Brettanomyces spp., Saccharomyces bailii and Saccharomycodes ludwigii were resistant to 500 μg SO_2 ml^{-1}. This could be due, at least in part, to sulphur dioxide acting as an oxygen scavenger thereby hindering oxidative phosphorylation (Beech and Thomas, 1985).

A recent study (Dalton, 1984) has shown that sausage yeasts with an oxidative metabolism or weak fermentative abilities are resistant to sulphite at a neutral pH (initially pH 7.0). Debaryomyces hansenii (the dominant yeast on sausages), Candida zeylanoides, Pichia membranaefaciens and Torulopsis candida have been found to produce acetaldehyde that binds 94-98% of the sulphite available (Table 13). Cryptococcus albidus grew well in sulphited broth although it did not produce acetaldehyde. So too with Rhodotorula rubra although the growth rate was retarded

Table 13. Percentage of sulphite bound by 6 yeast species grown in sulphited[§] lab lemco broth*

Yeast Species	Bound Sulphite** (%)
Debaryomyces hansenii	98
Pichia membranaefaciens	98
Candida zeylanoides	94
Torulopsis candida	95.5
Cryptococcus albidus	23
Rhodotorula rubra	23
Control (uninoculated)	22

* Adapted from Dalton (1984)

§ metabisulphite (500 µg SO_2 ml^{-1})

** Determined by the spectrophotometric method of Banks and Board (1982a).

(Dalton, 1984). This reflects the situation in sausages where sulphite favours the growth of Deb. hansenii thus reducing the numbers of Rhodotorula and Cryptococcus spp. (Dalton, 1984).

Previous work on sulphited meat products has been mainly on sausages, so when sulphited lamb products became available the present study was set up to determine if, as with sausages: (1) sulphite favoured the growth of yeasts, and (2) whether or not the loss of preservative occurred. Although it has been established that sulphite induces sausage yeasts to secrete acetaldehyde in a broth initially poised at pH 7 (Dalton, 1984), the influence of pH, sulphite concentration and substrate availability (as related to meat components) has not been studied. The present study was therefore undertaken to do so, with yeasts from minced lamb, and to establish whether non-acetaldehyde producing yeasts could also tolerate sulphite. Yeasts from the field environment and slaughterhouse were studied with the objective of establishing a contamination route.

MATERIALS AND METHODS

Route of Microbial Contamination

Survey of Microflora Associated with Sheep Pastures

Samples were collected aseptically in sterile stomacher bags (Sterilin) from sheep pastures during Summer, Autumn, Winter and Spring. Samples were taken from pasture plants, hay and soil together with samples of drinking water, faecal material and fleece.

Samples (20 g or 20 ml) were placed in 180 ml quarter-strength Ringer solution (Oxoid) in a stomacher bag which was then placed inside a second one before homogenising at high speed for 60 sec with a Colworth Stomacher (Lab Blender 400, Seward, London) at room temperature.

Samples (0.1 ml) of each tenfold dilution (prepared in quarter-strength Ringer solution) were spread onto Rose Bengal chloramphenical agar (RBC - Lab M) and Plate Count Agar (PCA - Lab M). Three replicates of each dilution were prepared and incubated at 20°C for 4 d (PCA) or 15°C for 5 d (RBC). The latter were wrapped in aluminium foil.

Survey of Microflora Associated with the Slaughterhouse

Swabs were taken - using a sterile cotton tipped applicator placed in 9 ml 0.5% peptone water (Patterson, 1968a) - from the neck, belly, tail and back regions of the lamb carcasses: (1) after slaughter; (2) after defleecing; and (3) after washing (Figs. 5,6). Fleece samples mainly from the head region, were collected

Figure 5.

LAMB CARCASS PROCESSING LINE

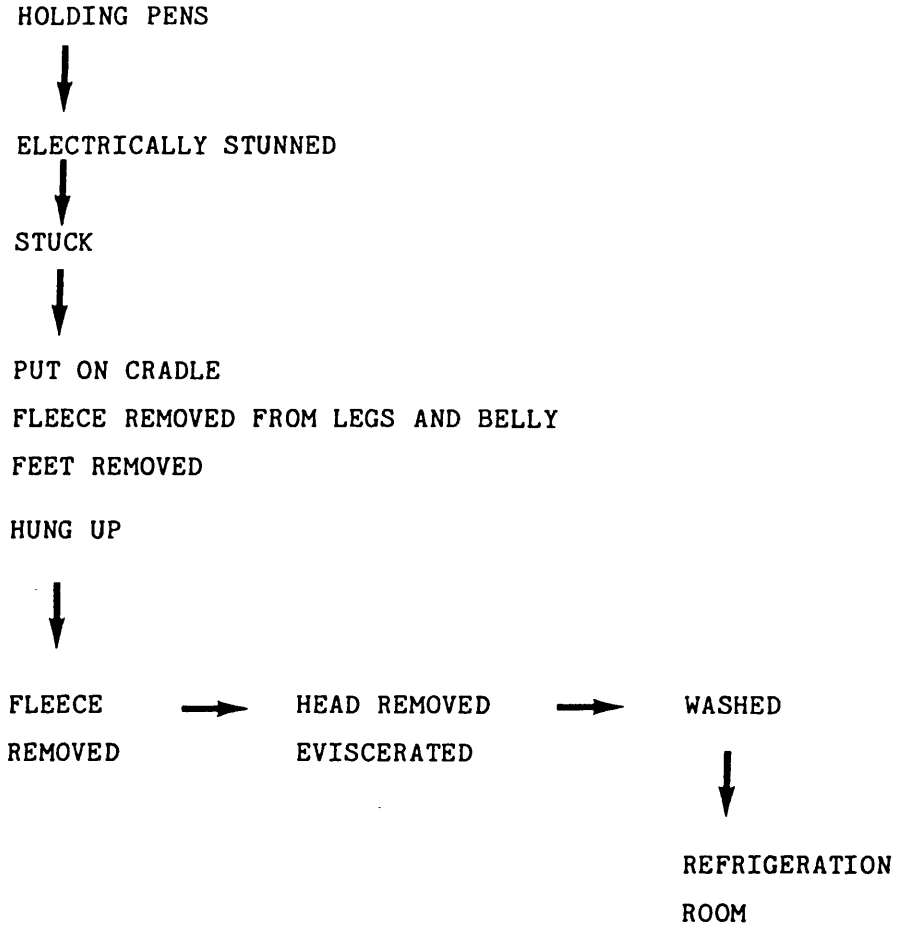
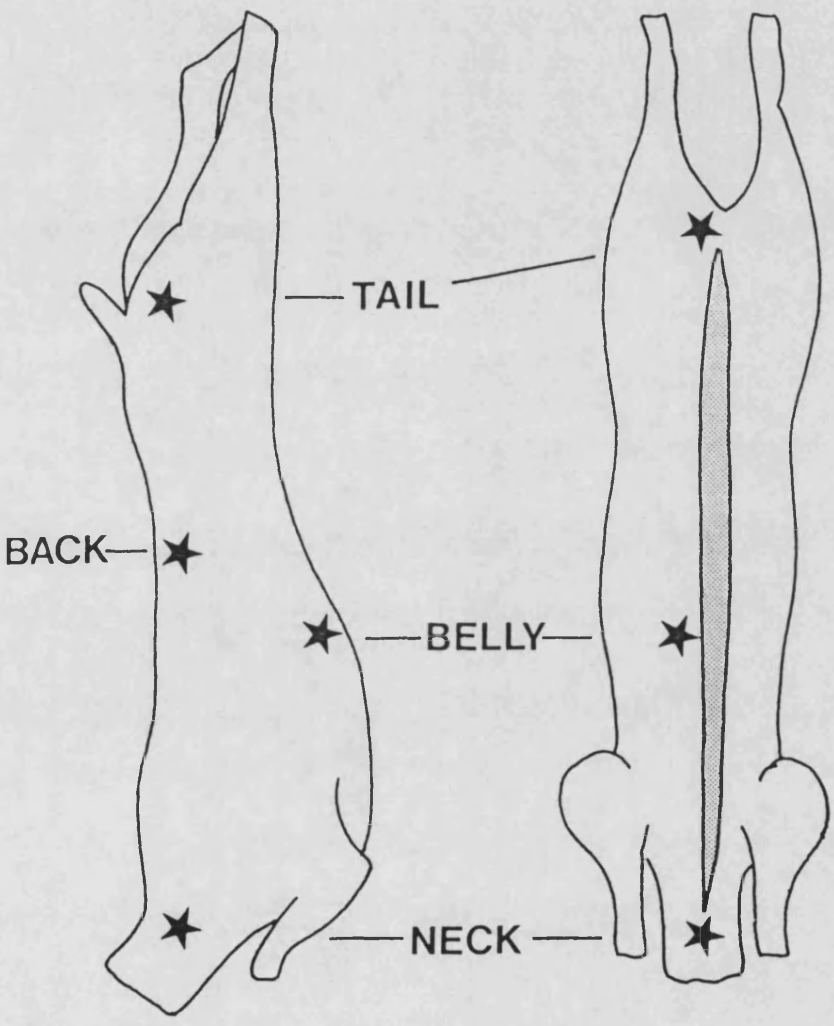


Figure 6

SAMPLING SITES ON LAMB CARCASS



post-slaughter. Swabs were taken from slaughtermen's aprons.

Tenfold dilutions were prepared with 0.5% peptone water and spread onto Pseudomonas selective medium (CFC - Mead and Adams, 1977), Plate Count Agar (Lab M) and Rose Bengal chloramphenicol agar (Lab M).

Meat Samples

Minced lamb, unsulphited and sulphited lamb products were obtained either from butchers' shops (wrapped in waxed paper or polythene bags) or from supermarkets (prepared in aluminium foil trays with cardboard covers or polystyrene trays covered with a thin plastic film).

Storage of Meat Samples

Minced lamb and lamb products were stored with access to air at 5°C or 15°C for up to 24 d. Samples were initially taken daily and subsequently at 2-4 d intervals.

Preparation of Sulphited Minced Lamb

As sulphite binds to cellulose acetate membrane filters (Banks, 1983; Stratford, 1983), sulphite solutions were prepared by the method of Dalton (1984) by direct addition of sodium metabisulphite (Analar, BDH) to sterile distilled water. Sterility of these solutions was tested by inoculation into 100 ml of modified lab lemco broth in Erlenmeyer flasks (250 ml capacity).

Addition of Sulphite to Minced Meat

One kg quantities of minced lamb were weighed in surface sterilised (70% alcohol) plastic bowls. A freshly prepared sulphite solution (50 ml) was added to the minced meat to give a final concentration of 500 μg SO_2 g^{-1}. Sterile distilled water (50 ml) was added to control samples (unsulphited meat). The meat was thoroughly mixed by hand (wearing surface sterilised Triflex gloves - Travenol). Samples (150 g) were placed in surface sterilised plastic trays (9 x 18 cm Thorpac, Glos.) covered with cellophane (British Cellophane) and heat sealed.

CHEMICAL ANALYSIS

Determination of Sulphite Concentration

Free, bound and total sulphite were determined by the method developed by Banks and Board (1982a).

Sample Preparation

A homogenate of meat sample was obtained by vigorously shaking (for 30 sec) a 5 g sample of meat with 20 ml of chilled deoxygenated distilled water (flushed with nitrogen and kept on ice) and glass beads in a screw cap bottle (50 ml capacity).

Free Sulphite Determination

The meat homogenate was placed immediately into a round bottom 250 ml flask with a magnetic stirrer. The flask had a centre neck and two angled side necks (FR 250/322A - Scientific Glass Laboratories Ltd., Stoke-on-Trent, Staffordshire). Nitrogen was

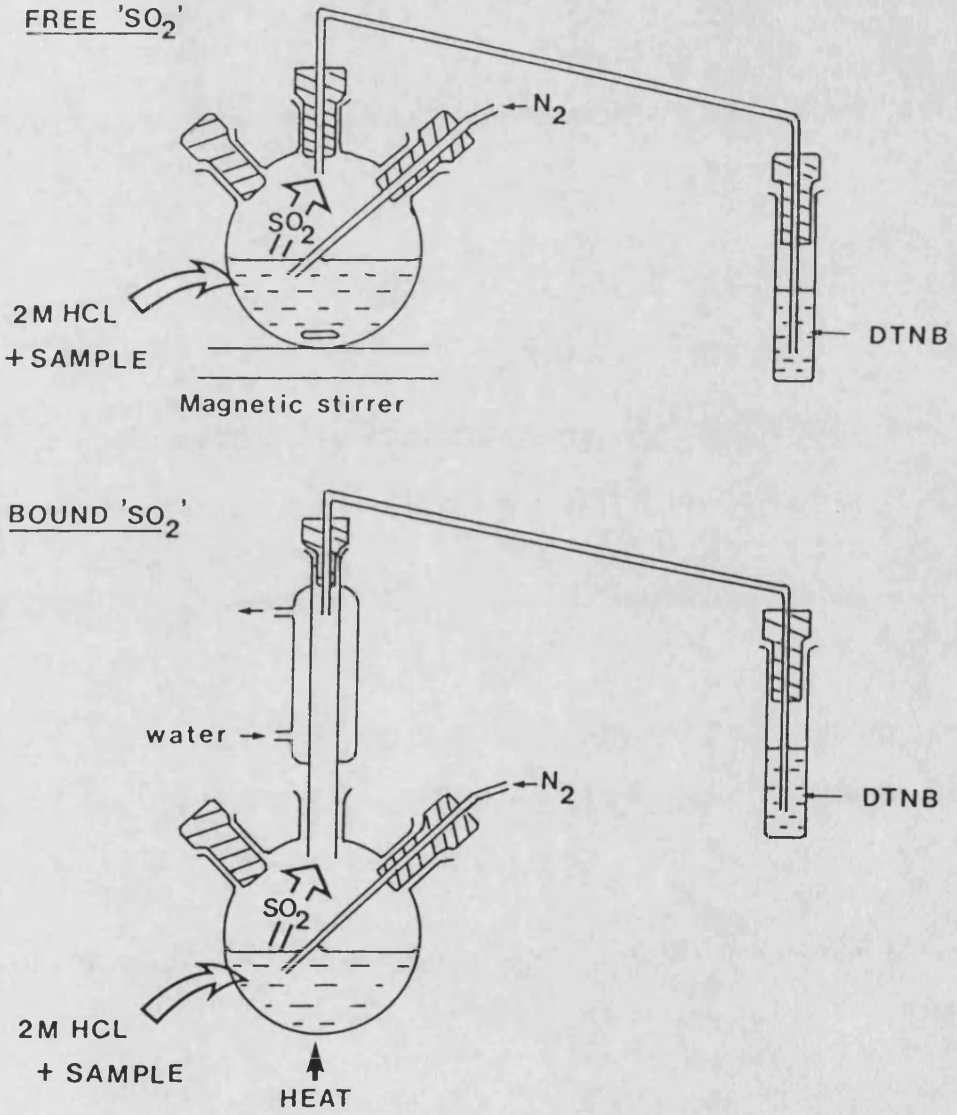
supplied (20 cm^{-3} s^{-1}; Air Products, Bristol) via a modified Dreschel bottle head (DMP 25/3 - with one side arm sealed by heating the glass) through a side neck (Fig. 7). Another modified Dreschel bottle head, was placed in the centre neck and connected to a second Dreschel bottle head with an elongated dip tube leading into a boiling tube (TT15/3). Thus the dip tube was placed below the surface of a solution (25 ml) of 5,5-dithiobios 2-nitrobenzoic acid (2.3 x 10^{-3} M, DTNB) dissolved in 2 M phosphate buffer (pH 8) containing ethanol (10% w/v). Hydrochloric acid (2 M, HCl, 20 ml) was added via the stoppered side neck (Fig. 7) and the glass stopper was replaced immediately into position. After 15 min the boiling tube was removed, phosphate buffer (25 ml) added to the DTNB solution and absorbance measured at 412 nm (Spectrophotometer SP6 550 UV/VIS, Pye Unicam). Absorbance was related to sulphite concentration by use of a standard curve obtained from sulphite solutions (containing starch granules) standardised by titration with iodine (0.01 N).

Bound Sulphite Determination

The flask containing meat homogenate was transferred to a heater bed and connected to a vertical condenser (C3/23 Quickfit, England) via the centre neck (Fig. 7). Nitrogen was supplied via a modified Dreschel bottle head in one side neck. A modified Dreschel bottle head was placed at the top of the condenser and connected to a second Dreschel bottle head in a boiling tube with its elongated dip tube below the surface of the DTNB (25 ml) solution. The sample was boiled for 15 min. The boiling tube was then removed, phosphate

Figure 7.

DIAGRAM OF THE APPARATUS USED TO DETERMINE FREE AND BOUND SULPHITE. *



* Adapted from Banks and Board (1982a)

buffer (25 ml) added to the DTNB solution and the absorbance read at 412 nm.

Total Sulphite Determination

The sum of the free and bound sulphite gave the concentration of the total sulphite present.

Determination of Total Sulphite by an Enzymatic Method

The concentration of total sulphite in lamb burgers was determined using the Boehringer Mannheim assay kit (catalogue number 725 854).

pH Determination

The pH of the aqueous layer of the meat homogenate (20 g meat sample homogenised in 180 ml quarter-strength Ringer solution) was determined using a pH meter (Pye Model 291, Pye Unicam).

MICROBIAL ANALYSIS

Sampling of Meat

Meat samples (20 g) consisted of minced meat taken from the edge and centre of meat products with a sterile scalpel.

The 20 g meat sample was placed in 180 ml of sterile, quarter-strength Ringer solution (Oxoid) in a sterile stomacher bag and homogenised for 60 sec in a Colworth Stomacher 400 (Seward, London). The homogenate was poured into a sterile screw cap bottle (500 ml capacity). Serial dilutions were prepared in quarter-strength Ringer solution.

Enumeration of Microorganisms

The following media were used to enumerate the microorganisms from meat products. Three replicates of appropriate dilutions were set up for each medium. Selectivity of each medium was checked by Gram-staining random colonies.

Total Viable Count (T.V.C.)

Samples (0.1 ml) of serial dilutions of meat homogenates were spread on Plate Count Agar (PCA, Lab M) and incubated at 20°C for 4 d.

Brochothrix thermosphacta

Samples (0.1 ml) of serial dilutions of meat homogenates were spread on Streptomycin thallous acetate actidione agar (STAA, Gardner, 1966) and incubated at 20°C for 3 d.

Pseudomonads

Samples (0.1 ml) of serial dilutions of meat homogenates were spread on centrimide-fusidic acid-cephalodrine agar (CFC, Mead and Adams, 1977) and incubated at 15°C for 48 h.

Lactobacilli

One ml samples of serial dilutions of meat homogenates were inoculated into 10 ml of molten (45°C) lactobacilli selective medium (Keddie, 1951). After setting, a 10 ml overlay of molten medium was added. Incubation was at 25°C for 5 d.

Enterococci

One ml samples of serial dilutions of meat homogenates were inoculated into 10 ml of molten (45°C) kanamycin aesculin azide broth (Oxoid) with 1.5% (w/v) agar. After setting a 10 ml overlay of molten medium was added. Incubation was at 37°C for 24 h.

Enterobacteriaceae

Lactose Fermenting Enterobacteriaceae

One ml samples of serial dilutions of meat homogenates were inoculated into 10 ml of molten (45°C) Violet Red Bile agar (VRBA, Lab M). After setting, a 10 ml overlay of molten medium was added. Incubation was at 37°C for 24 h.

Glucose Fermenting Enterobacteriaceae

One ml samples of serial dilutions of meat homogenates were inoculated into 10 ml of molten (45°C) Violet Red Bile glucose agar (VRBG, Oxoid). After setting a 10 ml overlay of molten medium was added. Incubation was at 30°C for 24 h.

Yeasts

An initial survey was conducted using 4 different media with incubation at 5°, 15°, 20° or 25°C and examination at 3, 5, 7 d except for those at 5°C, which were examined at 7, 10 and 14 d. A second survey was done using 8 media incubated at 15°C for 5 d. Six replicates were prepared for each medium.

1. Rose Bengal chloramphenicol agar (RBC, Lab M) wrapped in aluminium foil (to protect from light) during incubation.

2. Rose Bengal chloramphenicol agar with the addition of a filter sterilised solution of Dichloran (2, 6 dichloro-4-nitroaniline) to give a final concentration of 2 μg ml^{-1} (DRBC, King et al., 1979) wrapped in aluminium foil during incubation.

3. Oxytetracycline glucose yeast extract agar (OGYA, Oxoid, Mossel et al., 1970).

4. OGYA with the addition of a filter sterilised solution of Gentamicin to give a final concentration of 50 μg ml^{-1} (OGGYA; Mossel et al., 1970, 1975).

5. Malt extract agar acidified (pH 3.5) with 5 ml 10% lactic acid (w/v) to 100 ml medium (Holwerda, 1952; Jarvis, 1973).

6. Plate Count Agar acidified (pH 3.5) with one part 10% (w/v) citric acid to 10 parts medium (Dowdell and Board, 1968).

7. Malt extract agar with 2 ml of antibiotic solution added to 100 ml of medium (500 mg chlortetracycline HCl and 500 mg chloramphenicol in a 100 ml filter sterilised stock solution - Speck, 1976).

8. Plate Count Agar with 2 ml of stock antibiotic solution added to 100 ml of medium (as used with malt agar - Speck, 1976).

Subsequently 0.1 ml samples of serial dilutions of meat homogenates were spread on Rose Bengal chloramphenicol agar (RBC, Lab M) - wrapped in aluminium foil. Three replicates of each dilution were incubated at 15°C for 5 d. OGYA was used in conjunction with RBC when appropriate.

Purification of Yeast Isolates

Yeast cultures isolated from meat, slaughterhouse and field

samples were streaked onto Sabouraud's Dextrose Agar (SDA). Single isolated colonies from these primary plates were then purified by repeated replating (van der Walt and Yarrow, 1984) and incubated at 15°C for 5 d. The isolates were further subcultured onto Bijou slopes of SDA and incubated until good growth was obtained (3-5 d). The purity of the isolates was checked with a microscope. Stock cultures were then stored at 4°C.

Yeast Identification

Yeast isolates were identified according to the taxonomy proposed by Lodder (1970) and revised by Kreger-van Rij (1984).

Stock Cultures Tested

The following stock cultures were inoculated, in conjunction with the unknown yeast isolates, onto yeast identification media, and incubated at 25°C.

Candida famata (NCYC 611), C. ingens (NCYC 822), C. mesenterica (NCYC 390), C. rugosa (NCYC 351), C. valida (NCYC 327), Cryptococcus macerans (NCYC 578), Hansenula anomala (NCYC 711), Pichia fermentans (NCYC 850), Rhodotorula rubra (CMI 38784) and Torulopsis petrophilum (ATCC 20225).

Preparation of Inoculum

Yeast cultures were grown on starvation medium (Bacto yeast nitrogen base (Difco) with 1.5% (w/v) purified agar (Oxoid) and 0.1% (w/v) glucose - A.R. grade, Fisons) as suggested by Dalton (1984) and incubated at 25°C for 4 d. A yeast suspension was

prepared in sterile distilled water, centrifuged at ca. 2600 **g** for 20 min (Centaur 2, MSE centrifuge) and washed twice.

The inoculum was standardised by diluting with sterile distilled water until black lines drawn on a white card could be seen as diffuse bands, equivalent to +2 growth (approximately 10^6 cells ml^{-1} - Wickerham, 1951; Anon., 1986).

Macromorphology

The gross morphology was studied on 5% malt extract agar (5% (w/v) malt extract (Oxoid) and 1.5% (w/v) agar No. 2 - Lab M) (van der Walt and Yarrow, 1984). Pigmentation, presence or absence of extracellular polysaccharide, texture and shape of colony was recorded after 3 weeks incubation at 25°C (Dalton, 1984).

Pellicle, ring and sediment formation and appearance were studied in yeast morphology (YM) broth (Difco) cultures after 2, 7 and 21 d incubation at 25°C (Anon., 1986).

Micromorphology

The micromorphology was examined in YM broth after 2 d incubation at 25°C (Anon., 1986) and 5% malt extract agar cultures after 3 d incubation at 25°C (Dalton, 1984; van der Walt and Yarrow, 1984). Minimum and maximum length and breadth of cells were recorded using a microscope with a calibrated eyepiece graticule. Brightfield illumination was used with a x40 objective. Vegetative reproduction in the form of fission cells or budding (monopolar, bipolar or multilateral - van der Walt and Yarrow, 1984) was noted.

The formation of pseudohyphae, true mycelium, ballistospores, arthrospores, ascospores, endospores, chlamydospores and teliospores were determined by direct examination with a microscope of "Dalmau type plates" (yeast suspensions growing under a sterile coverslip) on corn meal agar (Oxoid) after 10 d incubation at 25°C (Wickerham, 1951; Dalton, 1984; van der Walt and Yarrow, 1984). Pseudohyphae were recorded as mycotorula, mycotoruloides, candida, mycocandida or blastodendrion in type (van der Walt and Yarrow, 1984).

Water suspensions were made of yeast cultures from corn meal agar; 5% (w/v) malt extract agar (Wickerham, 1951; Kurtzman, 1984), and from slopes of potassium acetate agar (1% (w/v) potassium acetate, 0.1% (w/v) glucose, 0.25% (w/v) yeast extract, 1.5% (w/v) agar - Anon., 1986) after 21 and 28 d incubation at 25°C and examined with a microscope for ascospore formation. Heat fixed preparations were stained by steaming with 5% (w/v) aqueous malachite green and counterstained with 0.5% (w/v) safranine (van der Walt and Yarrow, 1984).

Fermentation of Carbohydrates

One part (0.5 ml) of filter sterilised (Sartorius Minisart filters, 0.2 µm) 6.7% (w/v) Bacto yeast nitrogen base (Difco) and 20% (w/v) of the carbon source (glucose (A.R. grade Fisons), galactose, lactose, maltose or sucrose - Analar, BDH) or 40% (w/v) raffinose (van der Walt and Yarrow, 1984) was added to 9 parts (4.5 ml) sterile distilled water in Bijou bottles containing Durham tubes (25 mm x 6 mm).

Yeast suspensions (0.1 ml) were inoculated into the bottles, incubated at 25°C and examined for gas production at 2, 7, 14, 21 and 28 d.

Multipoint Inoculation

Centrifuged and washed inocula of yeast cultures were inoculated onto the following media by a Denley multipoint inoculator (Denley, Sussex). Twenty one isolates were inoculated in duplicate and incubated at 25°C and examined at 3, 5 and 7 d (Shifrine et al., 1954; Beech et al., 1955; Dalton, 1984).

Assimilation of Carbon Compounds

Assimilation of carbon compounds was tested with an agar modification (Dalton, 1984) of the method of Beech et al. (1968) and Anon.(1986). One part (10 ml) filter sterilised 6.7% (w/v) yeast nitrogen base (Difco) and 5% (w/v) of the carbon source were added to 9 parts (90 ml) of sterile 1.5% (w/v) purified agar (Oxoid) at 45°C and poured into Petri dishes. A positive control (with glucose) and a negative control (with no carbon source) were also included. Duplicate plates of each carbon source were inoculated for each set of yeast isolates being screened. The carbon sources tested at a final concentration of 0.2% (w/v) were: glucose, galactose, sorbose, sucrose, maltose, celliobiose, trehalose, lactose, melibiose, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, erythritol, ribitol, galactitol, D-mannitol, D-sorbitol, α-methyl glucoside, salicin, lactate, succinate, citrate and myo-inositol. Ethanol was used at a final concentration

of 0.62% (v/v), glycerol 0.4% (v/v), melezitose 0.36% (w/v) and raffinose 1% (w/v). All compounds were analytical grade reagents (BDH, Fisons or Sigma). Inulin (0.5 g) and starch (0.5 g) were dissolved by heating in 90 ml distilled water with 1.5% (w/v) purified agar. After autoclaving (121°C for 15 min) 10 ml of filter sterilised 6.7% (w/v) yeast nitrogen base (Difco) was added and the media were poured into Petri dishes.

Assimilation of Nitrogen Compounds

Assimilation of nitrogen compounds was tested with an agar modification (Dalton, 1984) of the method of Beech et al. (1968) and Anon. (1986). One part (10 ml) of filter sterilised 11.7% (w/v) yeast carbon base (Difco) and 0.78% (w/v) potassium nitrate (AR grade, Fisons) were added to 9 parts (90 ml) of sterile 1.5% (w/v) purified agar (Oxoid) and poured into Petri dishes. A control lacking potassium nitrate was included.

Growth on Sodium Chloride Medium

Growth on sodium chloride medium was tested by an agar modification of the method of Wickerham (1951). One part (10 ml) of filter sterilised 6.7% (w/v) yeast nitrogen base (Difco) was added to 9 parts (90 ml) of molten agar (45°C) containing 10% (w/v) sodium chloride, 5% (w/v) glucose and 1.5% (w/v) purified agar (Oxoid) and poured into Petri dishes.

Growth at Different Temperatures

Yeast inocula were multipoint inoculated onto 2% malt extract

agar (2% (w/v) malt extract, 1.5% (w/v) agar). Duplicate plates were incubated at 5°C for 10 d and 37°C for 5 d (Dalton, 1984).

Growth in Vitamin-free Medium

One part (1 ml) filter sterilised solution containing 16.7% (w/v) vitamin free yeast base (Difco) was added to 9 parts (9 ml) sterile distilled water in test tubes. Yeast suspensions (0.1 ml) were added to the test tubes and incubated at 25°C and examined for growth at 7, 14 and 21 d (Anon., 1986). Inocula were taken from tubes with growth and inoculated into a second vitamin free broth. Growth was only recorded as positive if the yeast grew in the 2nd tube (van der Walt and Yarrow, 1984; Anon., 1986).

Production of Urease

Five ml of filter sterilised 40% urea solution was added to 95 ml sterilised urea agar base (Oxoid) at 45°C and pipetted into 25-well replica dishes (10 x 10 cm - Sigma). Washed and standardised yeast suspensions were inoculated into each well (2 x 2 cm); and development of a pink colouration (due to phenol red indicator) after 24 h incubation at 25°C was recorded as urea hydrolysis.

Gelatin Hydrolysis

Fifteen grams of gelatin (Oxoid) and 0.5 g glucose (Wickerham, 1951) were dissolved by heating in 100 ml distilled water and autoclaved (121°C for 15 min). Molten gelatin was pipetted into 25-well replica dishes and cooled at 5°C. Washed, standardised

yeast suspensions were inoculated into each well and incubated at 15°C for 4 weeks. Liquefaction of gelatin was recorded as hydrolysis.

Starch Formation

Yeast cultures multipoint inoculated onto yeast nitrogen base with 1.5% (w/v) purified agar and glucose 0.2% (w/v) as the carbon source were flooded with iodine after 7 d incubation at 25°C (Wickerham, 1951). A blue-black colouration indicated extracellular starch and a brown colouration indicated extracellular glycogen.

Resistance to Damage by Photodynamic Effects

Yeast cultures were multipoint inoculated onto yeast nitrogen base with 1.5% (w/v) purified agar and 0.2% (w/v) glucose containing the following concentrations of Rose Bengal dye (tetra-iodo-tetrachlorofluorescein - Sigma): 1×10^{-3}, 1×10^{-4}, 1×10^{-5} and 1×10^{-6} mol l^{-1}, used as a singlet oxygen producer (Percival and Dodge, 1983; Banks et al., 1985a, b). Duplicate plates of each set of yeast cultures were either incubated wrapped in aluminium foil or continually illuminated (28 μmol m^{-2} s^{-1}) with a fluorescent light tube at 25°C.

Acetaldehyde Detection Medium

Yeast cultures were inoculated onto 25-well replica dishes or multipoint inoculated onto an agar devised to detect acetaldehyde producing yeasts. The medium was based on the principle of Endo (1914) agar but containing glucose instead of lactose (Brown,

1977). A modified lab lemco broth with 1.5% agar or glucose/peptone agar (1% (w/v) glucose, 1% (w/v) mycological peptone, 1.5% (w/v) agar) was used with Schiff's reagent (2.5 g l^{-1} sodium sulphite (BDH) and 0.4 g l^{-1} Basic fuchsin (BDH) dissolved in 4 ml 100% ethanol). Sodium sulphite added to the solution (before or after autoclaving) binds to basic fuchsin and thus renders the medium colourless (Margolena and Hansen, 1933). Other concentrations of sodium sulphite (0.31 - 2.50 g l^{-1}) and basic fuchsin (0.05 - 0.40 g l^{-1}) resulted in a faint-pink colouration of the medium.

The production of acetaldehyde was detected by restoration of the red colour in the medium (Neuberg and Nord, 1919; Margolena and Hansen, 1933). Petri dishes were incubated protected from the light (wrapped in aluminium foil) to avoid reddening at 25°C and examined at 1, 2, 3, 4 and 5 d.

Yeast cultures were inoculated onto medium containing sodium lactate (1% (v/v) - Sigma), as an alternative carbon source, and Schiff's reagent.

Samples (0.1 ml) of serial dilutions of meat homogenates were spread on oxytetracycline glucose yeast extract agar (Oxoid) containing Schiff's reagent.

The following chemicals (20 µl) were inoculated onto the acetaldehyde detection medium with and without adjustment to pH7.0: glucose, acetoin, D-glucose-6-phosphate, maltose, maltotriose, pyruvate, glycerol, 2-oxoglutaric acid, acetaldehyde, lactate, diacetyl, α-ketoglutaric acid and 1-undecene.

Sulphite Tolerance of Pure Yeast Cultures

Microplate Method

Yeast cultures were screened for sulphite tolerance by inoculation into plates containing 96 microtitre-wells (Sterilin). Growth was recorded as absorbance (at 600 nm) with a microplate reader (MR600 - Dynatech) after 0, 18, 24, 42, 48, 66 and 72 h incubation at 25°C. Microplates with lids were stored in surface sterilised large square plastic Petri dishes (23 x 23 cm) containing two layers of tissue paper saturated with sterile distilled water.

Each well contained a final volume of 0.2 ml. Double strength solutions were prepared of unsulphited or sulphited glucose or lactate lab lemco broths in citrate phosphate buffer (Dawson et al., 1969) at appropriate pH values and 0.1 ml was pipetted into each well. A 0.1 ml volume was prepared from a 10^{-2} serial dilution of an 18 h yeast culture (grown in glucose or lactate lab lemco broth and incubated at 25°C in a shaking water bath) and inoculated into each well. Eleven different concentrations of sulphite were used (0-500 µg ml^{-1}) and 8 pH values (pH 3.6-7.0). The first vertical row of wells were uninoculated controls to which 0.1 ml of sterile distilled water was added.

Determination of Sulphite Binding in Pure Yeast Cultures

Two yeast cultures isolated from sulphited lamb burgers were identified with Candida norvegica (positive on acetaldehyde detection medium) and Candida vini (negative on acetaldehyde detection medium) and subsequently tested for their ability to bind

sulphite and produce acetaldehyde in modified lab lemco broth (Dalton, 1984).

To 90 ml of sterile broth (0.3% (w/v) lab lemco powder (Oxoid) and 0.5% (w/v) mycological peptone - Lab M) was added 5 ml of glucose (AR grade, Fisons) solution (sterilised separately at 10 psi flash in water) to give a final concentration of 2% (w/v). Five ml of freshly prepared sodium metabisulphite (Analar, BDH) solution was added to give a final concentration of 500 $\mu g\ ml^{-1}$ sulphite. Alternatively, 5 ml of sterile distilled water was added to the unsulphited broths.

A 1 ml sample was prepared from an 18 h (exponential phase) broth culture and inoculated into 100 ml of sterile broth (as above) in an Erlenmeyer flask (250 ml capacity) with a gauze covered cotton wool bung and incubated for 20 h or 24 h in a shaking water bath (90 oscillations min^{-1}) at 25°C. One ml of sterile distilled water was added to the uninoculated control.

The broths were unbuffered or buffered at pH 4, 5, 6 and 7 using the following buffers: Potassium citrate phosphate buffer and sodium citrate phosphate buffer (Dawson *et al.*, 1969) were set up at pH 4, 5, 6 and 7. Citric acid/sodium citrate buffer (Dawson *et al.*, 1969) was used at pH 4, 5 and 6. Succinic acid/sodium hydroxide buffer (Dawson *et al.*, 1969) was tested at pH 4 and 5. Three non-metabolisable buffers were also used: MES (2[N-Morpholine] ethane sulphonic acid - Sigma) at pH 6, HEPES (N-2-Hydroxyethyl-piperazine N'-2-ethane sulphonic acid - Sigma) at pH 7 and MOPS (3-[N-Morpholine] propane sulphonic acid - Sigma) at pH 7.

Subsequently sodium citrate phosphate buffer (0.1 M) was used in unsulphited and sulphited broths at pH 4, 5, 6 and 7. The pH was checked routinely with a pH meter (Pye Model 291, Pye Unicam).

A standard inoculum (1 ml) and a larger inoculum (giving a final concentration of ca 10^5 and 10^7 c.f.u.ml^{-1} respectively) of Candida norvegica and C. vini were inoculated into unsulphited and sulphited (500 µg $SO_2 ml^{-1}$) glucose and lactate lab lemco broths buffered at pH 4, 5, 6 and 7.

A standard inoculum (1 ml) of Candida norvegica and C. vini was inoculated into glucose lab lemco broth (pH 4, 5, 6 and 7) supplemented with sulphite (0, 100, 250, 500, 750 and 1000 µg SO_2 ml^{-1}).

Candida norvegica (the sulphite binder) was inoculated into unsulphited and sulphited lab lemco broth (pH 6) supplemented with various carbon sources. Five ml of the following solutions (sterilised separately at 10 psi flash in water) were added to give final concentrations of 0.5-2% (w/v): acetate, cellobiose, glycerol, maltose, sorbose, succinate, sucrose, starch, pyruvate (BDH), fructose, lactate, sorbitol, salicin (Sigma) and 3-O-methyl-D-glucose (3-O-methyl-D-glycopyranose - Sigma). Broth cultures were also supplemented with ethanol (filter sterilised) or with glucose (0.1-2% w/v).

Candida norvegica was inoculated into unsulphited or sulphited lab lemco broth buffered at pH 4, 5, 6 and 7 and supplemented with fructose or ethanol.

A 1 ml inoculum of C. norvegica originating from either lab lemco broth (without a carbon source) or from a supplemented broth

(glucose or lactate) was inoculated into unsulphited or sulphited glucose or lactate broth at pH 4, 5, 6 and 7. Subsequently inoculum from unsupplemented or supplemented broth (glucose, lactate, fructose or ethanol) was inoculated into unsulphited or sulphited pH 5 broth with or without a carbon source.

Zygosaccharomyces bailii

Broth (0.3% (w/v) yeast extract, 0.5% (w/v) mycological peptone) was buffered at pH 4, 5, 6 and 7 with potassium or sodium citrate phosphate buffer. To 90 ml of sterile broth was added 5 ml of glucose solution (to give a final concentration of 2% w/v) and 5 ml of freshly prepared potassium metabisulphite (Analar, BDH) solution to give a final concentration of 100 μg SO_2 ml^{-1}. Five ml of sterile distilled water was added to the unsulphited broths.

A 1 ml sample was prepared from a 18 h broth culture of Zygosaccharomyces bailii (NCYC 1427) and inoculated into 100 ml of sterile broth (as above) in an Erlenmeyer flask, and incubated for 21 h at 25°C in a shaking water bath.

Cell Density

One ml samples were removed (aseptically) immediately after inoculation and at 20 h or every 2 h in a 24 h incubation period. Cell density was determined by relating absorbance at 600 nm (Pye Unicam, SP6 550 UV/VIS) to viable cell number (colony forming units) from a standard curve.

Determination of Free and Bound Sulphite

One ml samples were aseptically removed immediately after inoculation and at 20 h or every 2 h in a 24 h incubation period. The concentration of free and bound sulphite was determined by the method of Banks and Board (1982a).

Acetaldehyde Determination

The concentration of acetaldehyde in the broth cultures was determined using the Boehringer Mannheim assay kit, catalogue number 668613. Sensitivity of the assay kit was tested with standard solutions of acetaldehyde (with and without added sodium metabisulphite).

Sulphate Test

The concentration of sulphate in the broth cultures was determined by a $BaCl_2$-EDTA titration (Anon., 1973; Sharma *et al.*, 1987). Broth culture (2.5 ml) was placed in a conical flask (50 ml capacity) and 1 ml of 0.5 N HCl added, followed by 10 ml of 0.04 N $BaCl_2$, and mixed. A buffer was prepared by adding 4.125 g of NH_4Cl to 56.5 ml of ammonia (sp. gr. 0.88) and the volume made up to 500 ml with distilled water. In addition to this 3.72 g of ethylenediaminetetra-acetic acid (EDTA) disodium salt and 2.03 g $MgCl_2.6H_2O$ were then added.

A total hardness-indicator tablet (BDH) was dissolved in 3 ml of buffer and then added to the sample. The sample was titrated against 0.005 N EDTA solution (disodium salt) until the red-brown colour changed to blue-grey. Standard sulphate solutions were set

up to relate volume of EDTA titrated to sulphate concentrations.

Dry Cell Weights

Candida norvegica and Candida vini were inoculated into 100 ml of unsulphited and sulphited (500 µg SO_2 ml^{-1}) lab lemco glucose broths buffered at pH 4, 5, 6 and 7 in Erlenmeyer flasks and incubated at 25°C in a shaking water bath (90 oscillations min^{-1}). After 20 h, the cultures were centrifuged at 2600 **g** for 20 min (MSE centrifuge), the yeast cells were resuspended in 10 ml volumes of 0.015 M NaCl solution (Herbert et al., 1971) and transferred into clean, dry, weighed conical glass centrifuge tubes with loose fitting metal caps. The yeast suspensions were centrifuged and washed two further times to remove solutes. After the final centrifugation most of the liquid was decanted to leave a small volume (< 0.5 ml) over the pellet. The tubes and their contents were dried to a constant weight (18 h) in a hot air oven at 103°C, cooled in a desiccator and weighed on an analytical balance sensitive to 0.1 mg.

Cell Size

Candida norvegica and Candida vini were inoculated into 100 ml of sulphited (500 µg SO_2 ml^{-1}) and unsulphited lab lemco glucose broths buffered at pH 4, 5, 6 and 7 in Erlenmeyer flasks and incubated at 25°C in a shaking water bath (90 oscillations min^{-1}). After 20 h suspensions were made of each yeast culture and width and length measurements made of 50 cells with the aid of a microscope with a calibrated eyepiece graticule.Brightfield illumination was used with a x40 objective.

Analysis of Total Carbohydrates in Yeast Cells

Candida norvegica was inoculated into 100 ml of lab lemco broth (without carbohydrate) and lab lemco broth supplemented with glucose in Erlenmeyer flasks incubated at 25°C in a shaking water bath. After 20 h the yeast suspensions were diluted with distilled water until both gave an absorbance reading at 600 nm (Pye Unicam, SP6 550 UV/VIS) (equivalent to 190 $\mu g\ ml^{-1}$ dry cell wt). A 0.2 ml sample of yeast suspension was pipetted into a thick walled glass tube and 0.2 ml of 5% (w/v) phenol solution (in water) added, and mixed. One ml of concentrated sulphuric acid (Analar, BDH) was rapidly added to the mixture and mixed immediately with a Whirlimixer (Fisons) and left for one hour at room temperature. Absorbance was read at 490 nm against a water blank (Herbert et al., 1971).

Yeast suspensions of known absorbance values (equivalent to 190 $\mu g\ ml^{-1}$ dry cell wt) were further analysed by extracting the low molecular weight components of the cells. The yeast suspensions were centrifuged at 2600 **g** for 20 min and the pellet extracted with 10 ml of 0.5 M trichloroacetic-acid (TAA) for 30 min on ice. These were then centrifuged and 0.2 ml of the decanted TAA was analysed for total carbohydrates. The pellet was resuspended in 10 ml of distilled water and 0.2 ml of this was analysed for total carbohydrates by the phenol method (as above).

Absorbance values were related to the standard curve obtained from solutions of glucose at concentrations of 0.001 mg to 0.1 $mg\ ml^{-1}$.

RESULTS

Yeast Identification

It was evident from the literature review (Section 1), that the contamination route of yeasts from pastures, *via* the fleece to the carcass and meat products has not been investigated in detail. More particularly the ratio of yeasts to bacteria, the dominant spoilage agents, has been mainly ignored. Attention was given to identifying the yeasts along the infection route and to this ratio in the present study in order to determine the extent, if any, to which sulphite influenced yeast contaminants in preserved lamb products.

Randomly selected colonies from yeast isolation medium were selected and purified by repeated re-streaking. Yeasts isolated in this way from minced lamb and lamb products (n = 117) and from field and slaughterhouse environments (n = 55) were characterised by macromorphology, micromorphology, fermentation and assimilation tests. The majority the yeasts were species of *Candida* (including *C. famata* formerly *Torulopsis candida*), *Cryptococcus* and *Rhodotorula*. The one exception to this, was *Trichosporon cutaneum*.

Additional biochemical tests were set up in conjunction with the conventional methods with 98 representative yeasts from pastures, carcass surfaces, minced lamb and lamb products. It is noteworthy that the majority of these yeasts grew at 5°C (Table 14) and hence have the potential of growth at refrigeration temperatures used for meat storage.

1. Route of Microbial Contamination

Four yeast isolation media were compared (Tables 15, 16a,b) in

Table 14. Characteristics of yeast isolates.

	MEAT	CARCASS	FIELD
Pellicle	13	0	10
Carotenoid pigment	12	11	40
Ascospores*	0	0	0
Arthrospores	3	0	0
Pseudohyphae	45	35	20
True hypha	3	9	0
Ferment glucose	14	0	10
Assimilate			
Glucose	100	100	100
Galactose	44	58	80
Sorbose	61	28	80
Sucrose	71	96	90
Maltose	44	69	90
Cellobiose	39	60	60
Trehalose	57	62	80
Lactose	14	28	30
Melibiose	21	12	10
Raffinose	40	51	90
Melizitose	47	55	90
Inulin	3	0	0
Starch	3	0	10
D xylose	41	53	90
L arabinose	35	40	80
D arabinose	34	53	50
D-ribose	48	44	40
L rhamnose	5	28	40
Ethanol	70	88	70
Glycerol	66	78	80
Erythritol	36	12	10
Ribitol	32	36	70
Galactitol	15	24	10
D mannitol	87	44	70
D sorbitol	72	42	80
α M glucoside	21	48	70
Salicin	38	44	90
Lactic acid	47	53	30
Succinic acid	35	36	50
Citrate	63	32	30
Inositol	21	24	30
Nitrate	14	8	30

* Ascospores were observed with stock cultures of Hansenula anomala (NCYC 711) and Pichia fermentans (NCYC 850).

Table 14. continued

	MEAT	CARCASS	FIELD
Growth			
Vitamin free	10	0	10
10% NaCl	76	48	60
5°C	94	100	90
37°C	6	0	0
Hydrolysed			
Urea	27	36	60
Gelatin	16	0	0
Production			
Starch	21	24	30
Acetaldehyde	38	12	50
Resistance to damage			
by photodynamic effects	9	16	33
Number of yeasts tested	63-117	25-45	10

Table 15. Comparison of different media for recovery of yeasts from sulphited lamb toppers[1].

	MAA			PCAA			OGYA			RBC		
°C	7**	10	14	7	10	14	7	10	14	7	10	14
5	2.83[§] ±0.90	2.83 ±0.90	3.00 ±0.90	0.67 ±0.20	0.83 ±0.17	1.00 ±0.26	14.50 ±1.50	19.50 ±2.60	22.00 ±2.60	12.33 ±2.03	15.00 ±2.60	16.00 ±3.50
	3**	5	7	3	5	7	3	5	7	3	5	7
15	1.50 ±1.50	6.70 ±1.30	6.40 ±1.47	1.67 ±0.42	2.67 ±0.80	2.67 ±0.84	25.00 ±2.70	26.50 ±2.20	27.30 ±2.75	17.80 ±2.50	18.30 ±2.60	18.80 ±2.20
20	4.50 ±1.48	5.00 ±1.50	5.67 ±1.80	2.33 ±1.02	3.33 ±1.60	3.30 ±1.40	25.20* ±3.62	18.20 ±2.18	16.30 ±1.90	11.30 ±2.04	11.70 ±1.90	12.67 ±1.90
25	6.66 ±2.90	7.50 ±1.56	8.33 ±1.05	1.30 ±0.56	3.50 ±2.50	3.00 ±0.63	23.70* ±2.50	16.00 ±1.40	16.50 ±1.20	15.50 ±1.50	15.70 ±1.60	16.20 ±1.90

[1] Statistical analysis (Sokal and Rohlf, 1969)

§ Mean c.f.u. x 10^2 g^{-1} (± standard error); 6 replicates of each dilution

* Higher counts at 3 d included bacterial colonies ** Days

MAA - Acidified Malt Agar (Holwerda, 1952; Jarvis, 1973)
PCAA - Acidified Plate Count Agar (Dowdell and Board, 1968)
OGYA - Oxytetracycline glucose yeast extract agar (Oxoid) (Mossel et al., 1970)
RBC - Rose Bengal Chloramphenicol agar (Lab M) incubated in the dark

Table 16a. Significant differences between mean c.f.u. after incubation at 5*, 15, 20 and 25°C for 3, 5 and 7 d.

Media	Incubation (d)					
	3		5		7	
OGYA	F = 0.18	25 = 20 = 15 §	F = 7.98	25 = 20 < 15	F = 9.10	5 = 20 = 25 < 15
	NSD		SD		SD	
RBC	F = 2.50	20 = 25 = 15	F = 2.50	20 = 25 = 15	F = 2.30	5 = 20 = 25 = 15
	NSD		NSD		NSD	
MAA	F = 0.73	15 = 20 = 25	F = 0.73	15 = 20 = 25	F = 2.89	15 = 5 = 20 = 25
	NSD		NSD		NSD	
PCAA	F = 0.50	15 = 20 = 25	F = 0.75	20 = 25 = 15	F = 1.70	15 = 5 = 20 = 25
	NSD		NSD		NSD	

P and F values obtained from analysis of variance (ANOVA)
NSD no significant difference when $P > 0.05$
SD significantly different when $P < 0.001$

§ When ANOVA gives significant difference, the differences between means were tested with the multiple range test (differences were significant at level of $P \leqslant 0.05$)
* 5°C only tested for 7 d incubation

Table 16b. Significant differences between the highest mean c.f.u. from each medium.

PCAA	MAA	RBC	OGYA
7d 20°C	7d 25°C	7d 15°C	7d 15°C
§ 3.30	= 8.33	< 18.80	< 27.30
*	P < 0.001	F = 29.00	

§ Multiple Range Test

* Analysis of Variance

Mean c.f.u. x 10^2 g^{-1}

a preliminary investigation, for efficiency in recovery of yeasts from sulphited lamb toppers (minced lamb with a pastry cover) with incubation at 15°, 20°, 25°C for 7 d and at 5°C for 14 d. Temperature did not appear to have significant influence on the selectivity of these media with the exception of oxytetracycline yeast extract agar (OGYA). In this instance the number of colony forming units recovered at 15°C were significantly higher than at other temperatures (Tables 15, 16a).

A comparison of the highest mean colony counts for the 4 media revealed that acidified Plate Count Agar (PCAA) and acidified Malt Agar (MAA) were significantly lower than those on Rose Bengal chloramphenicol agar (RBC) or OGYA (Tables 15, 16b). The number of yeasts recovered on OGYA was significantly greater than those on RBC (Tables 15, 16b). With the former, however, bacterial colonies were difficult to distinguish from those of yeast.

In a second survey, 8 media were compared for efficiency in recovery of yeasts from sulphited lamb burgers, with incubation at 15°C for 5 d. The acidified (MAA, PCAA) gave significantly lower yeast counts than the other media (Table 17). When MA or PCA was supplemented with antibiotics, however, the number of yeasts recovered were equal to those on OGYA and RBC.

Rose Bengal chloramphenicol agar (wrapped in aluminium foil to protect against photodynamic effects) was subsequently used as the yeast isolation medium because: 1) it detected significantly more yeasts than MAA or PCAA; 2) it gave counts equal to those on RBC + Dichloran, OGYA + Gentamicin, OGYA and MA + antibiotics; and 3) bacterial colonies were easily distinguished from those of yeasts.

Table 17. Comparison of 8 different media for recovery of yeasts from sulphited lamb burgers.

MAA		PCAA		DRBC		OGGYA		RBC	OGYA		MAant		PCAant
12.50 [§]	<	39.80	<	48.80	=	50.00	=	52.17	60.20	=	60.70	=	69.70
±2.40		±4.00		±13.40		±3.10		±3.30	±6.30		±2.40		±2.80

F = 25.5
P = <0.001
} Analysis of Variance

mean c.f.u. x 10^4 g^{-1} (± standard error)
§ Multiple Range test

_____ Not significantly different
MAA - acidified Malt Agar
PCAA - acidified Plate Count Agar
RBC - Rose Bengal Chloramphenicol agar - incubated in the dark
OGYA - oxytetracycline glucose yeast extract agar
OGGYA - OGYA with Gentamicin (Mossel et al., 1970, 1975)
DRBC - RBC with Dichloran (King et al., 1979) incubated in the dark
MAant, PCAant } Supplemented with Chlortetracycline and Chloramphenicol

Incubation at 15°C for 5-7 d was found to be the most suitable regime for recovery of yeasts from lamb products.

a) In the Field

The sheep pastures analysed in this study were situated on Salisbury Plain. The fields were unsheltered and at times of sampling, mist, rain and snow (in January) prevailed. In these wet and muddy conditions, the sheep were enclosed in pens and fed on grass or turnip stubble supplemented with hay.

Analysis of the sheep pastures throughout the year (Figs. 8b,9) revealed that the yeast populations (2.5×10^2 - 4.3×10^7 g^{-1} wet wt) were very small (0.001 - 2.6%) when compared with the total viable counts (4.0×10^5 - 2.2×10^{10} g^{-1} wet wt) - Table 18a. A sample of green turnip leaves, collected in January from an unstocked field, was the only exception. It harboured a yeast population (1.6×10^5 g^{-1} wet wt) of 40% when compared to the exceptionally low total viable count (4.0×10^5 g^{-1} wet wt). In contrast the yellow turnip leaves in the same field, were extensively contaminated with a microflora of 4.3×10^7 g^{-1} wet wt (yeasts) and 8.2×10^8 g^{-1} wet wt (total viable count).

Hay contained in a feeder (in March) was another site of high contamination. It had an exceptionally high total viable count (2.2×10^{10} g^{-1} wet wt) although the yeasts accounted for only 0.004% (9.5×10^5 g^{-1} wet wt).

The proportions of carotenoid-pigmented yeasts varied seasonally. They accounted for 5% of the yeast population in hay in December and 87% of the yeast flora in March (Fig. 8a). The same

Figure 8. The recovery of yeasts from field samples:

T	Turnips	S	Soil
G	Grass	F	Faecal material
H	Hay	W	Water
St	Straw	Fl	Fleece

a) Pigmented yeasts as a percentage of total yeasts

D - December

J - January

M - March

Jl - July

O - October

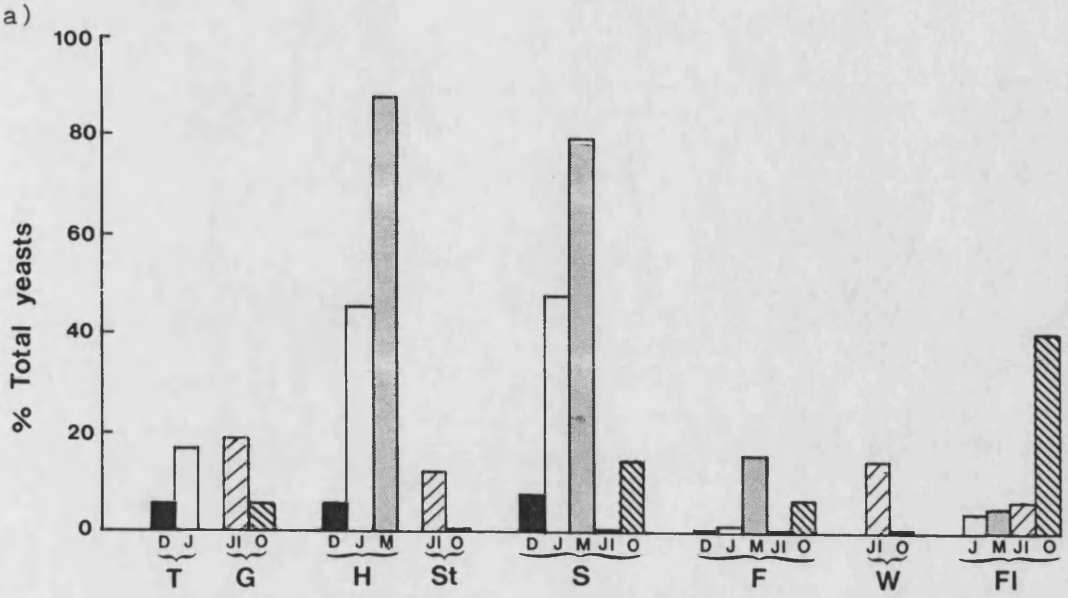
b) In Winter (December)

a	green leaves	d	stubble
b	yellow leaves	e	feeder
c	roots	f	ground

 Unstocked

 Stocked

a)


% Total yeasts
100
80
60
40
20
0
D J
JI O
D J M
JI O
D J M JI O
D J M JI O
JI O
J M JI O
T
G
H
St
S
F
W
Fl

b)

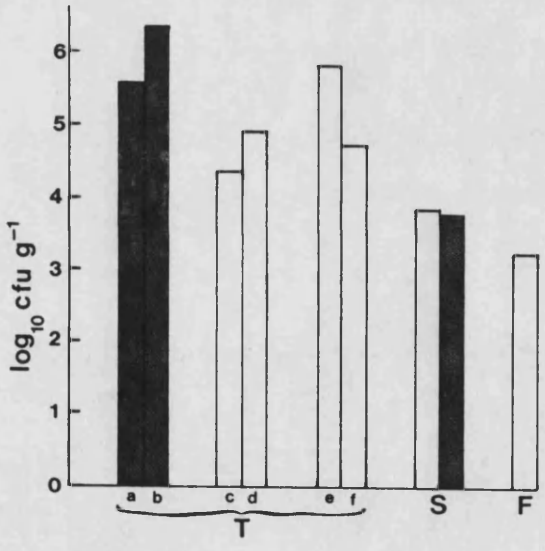

log10 cfu g-1
6
5
4
3
2
1
0
a b
c d
e f
T
S
F

Figure 9. The recovery of microorganisms from field samples:

a) In Winter (January)

b) In Spring (March)

c) In Summer (July)

d) In Autumn (October)

U Unstocked

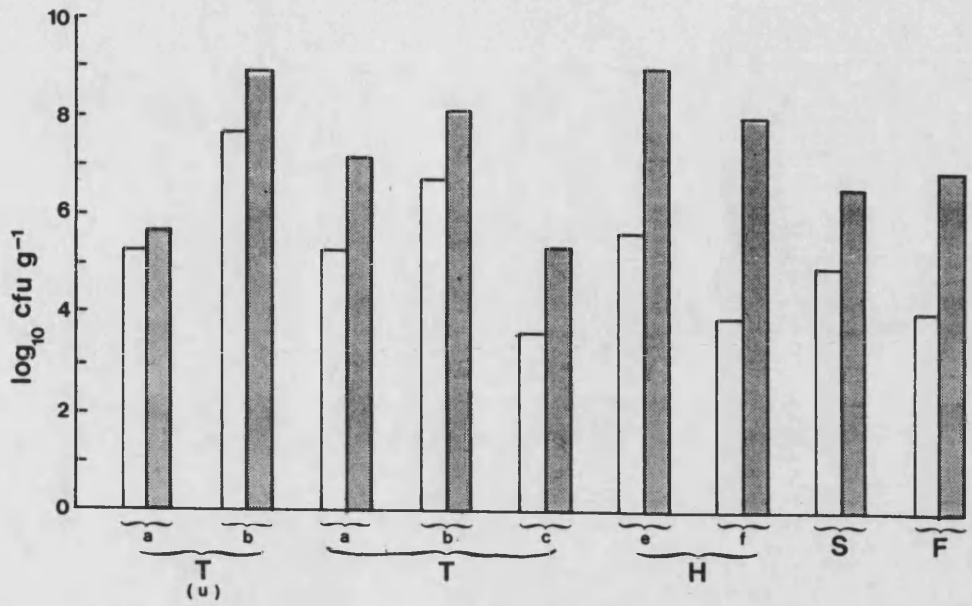
(Letters as Fig. 8)

Yeasts

Total Viable Count

Average of 2-5 samples

a)


10
8
6
4
2
0
log10 cfu g-1
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b
c
e
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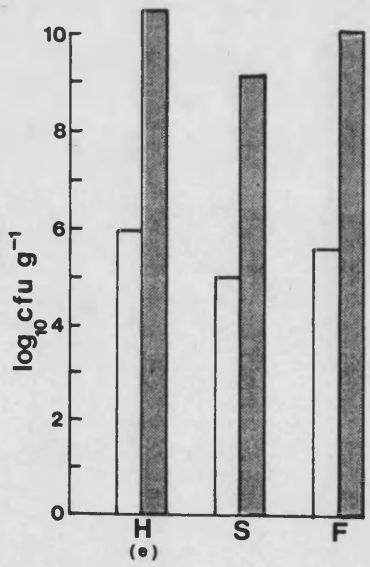
H

b)


10
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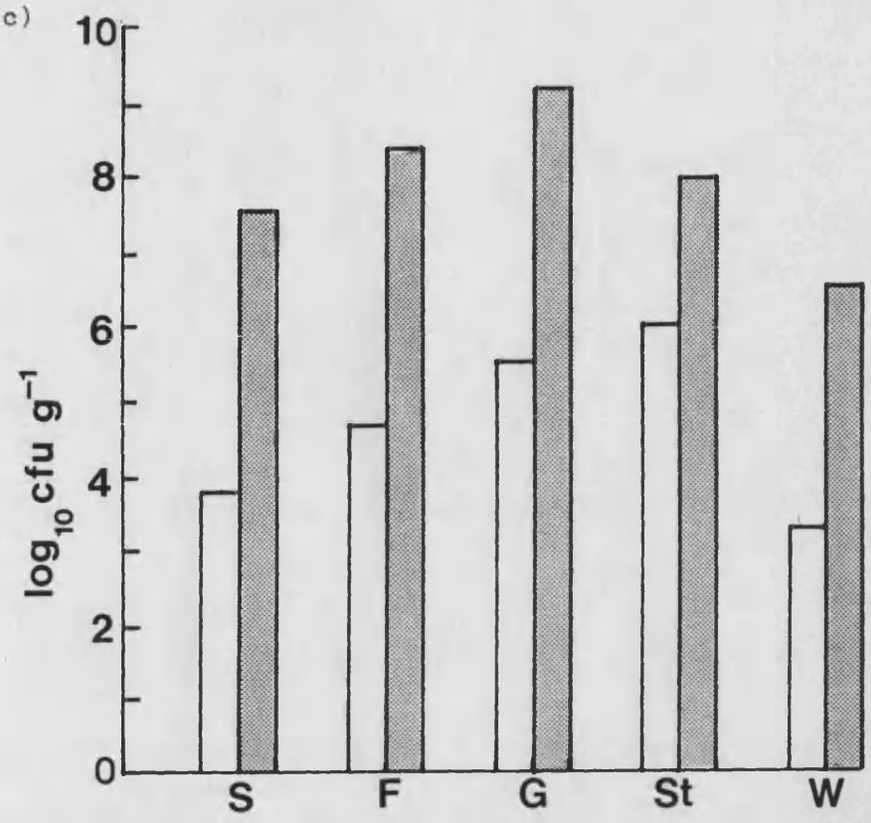
log10 cfu g-1
H
(e)
S
F

c)



d)

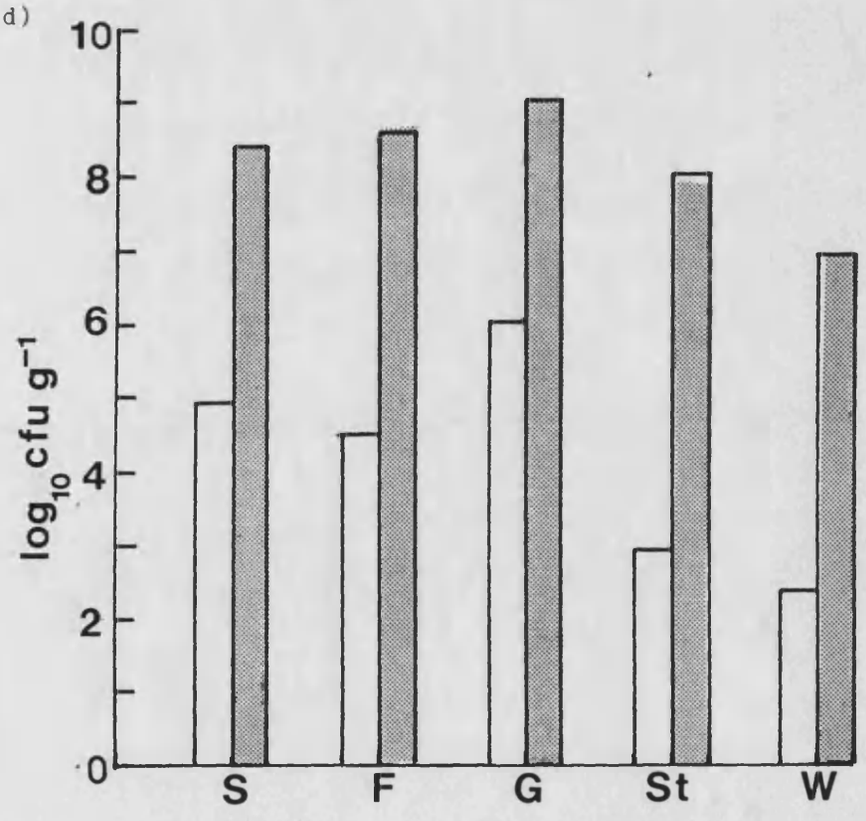


Table 18. The size of the Yeast Population as compared to the Total Viable Count.

a) In the Field Samples*

	January	March	July	October
Vegetation				
Grass	-§	-	0.02	0.14
Turnips	2.35**	-	-	-
Hay	0.025	0.004	-	-
Soil	2.60	0.01	0.014	0.041
Straw	-	-	0.071	0.001
Water	-	-	0.057	0.004
Faecal material	0.14	0.004	0.023	0.01
Fleece	1.50	12.67	0.002	4.54

b) In the Slaughterhouse samples

	Winter	Spring	Summer	Autumn
After defleecing				
Back	-	NA	NA	NA
Belly	0.90	2.03	0.09	0.351
Tail	-	0.73	0.057	0.222
After washing				
Back	-	1.13	0.49	1.303
Belly	0.25	-	0.141	0.723
Tail	-	2.89	0.275	2.299
Neck	-	0.74	-	-
Slit	-	5.00	-	-
Drip water	5.93	1.91	-	-
Butchers' aprons	0.35	0.20		
Fleece (head)	6.90	0.35	-	-

* Yeasts (isolated on RBC) as % of TVC (isolated on PCA)
- § Not sampled
** Average not including 40% on unstocked green turnip leaves
NA Not applicable as low counts of yeasts and TVC

trend was evident with soil samples; pigmented organisms accounted for 7.5% of the yeast population in December and 80% in March. A seasonal variation in the proportions of red-pigmented yeasts was recorded also by di Menna (1959).

The non-pigmented yeasts in the field samples were identified with Candida and Cryptococcus and the pigmented ones with Rhodotorula spp. or Cryptococcus infirmo-minatus (the imperfect state of Rhodosporidium infirmominatum) - Table 19.

Resistance to Damage by Photodynamic Effects

The ability of yeasts to survive on the fleece in the field environment and hence to subsequently contaminate the meat product via the carcass may depend, in part, on their resistance to damage by photodynamic effects. The non-pigmented yeasts isolated from the field, slaughterhouse and lamb products failed to form colonies on Rose Bengal (1×10^{-5} mol l^{-1}) medium exposed to continual illumination (28 µmol $m^{-2}s^{-1}$). The only isolates resistant to damage by photodynamic effects were the carotenoid-pigmented yeasts identified with Rhodotorula spp., mainly Rh. rubra (Plate 1). The pigmented Cryptococcus infirmo-minatus was not resistant.

b) On the Fleece

Analysis of the microflora on fleece samples taken from the field also showed seasonal variation in the numbers of yeasts vis à vis those of bacteria (Fig. 10, Table 18a). Thus yeasts accounted for 12.67% (3.8×10^{6} g^{-1} wet wt) of the total viable count (3.0×10^{7} g^{-1} wet wt) in March but only 0.002% (1.27×10^{3}

Table 19. Yeast species isolated along the contamination route.

	Species
Field:	Candida famata
	Candida sake
	Cryptococcus albidus var. albidus
	Cryptococcus infirmo-minatus
	Rhodotorula rubra
Fleece:	Candida spp.
	Cryptococcus spp.
	Rhodotorula rubra
Slaughterhouse:	
Carcass after defleecing:	
	Candida curvata
	Candida mesenterica
	Cryptococcus albidus var. albidus
	Cryptococcus laurentii
	Rhodotorula rubra
Carcass after washing:	
	Candida curvata
	Candida famata
	Candida glabrata
	Candida mesenterica
	Cryptococcus laurentii
	Rhodotorula minuta
	Rhodotorula rubra
Butchers' aprons:	
	Candida mesenterica
	Cryptococcus albidus var. albidus
	Rhodotorula rubra

Plate 1. The resistance of yeasts on Rose Bengal (1×10^{-5} mol l^{-1}) agar to damage due to photodynamic effects.

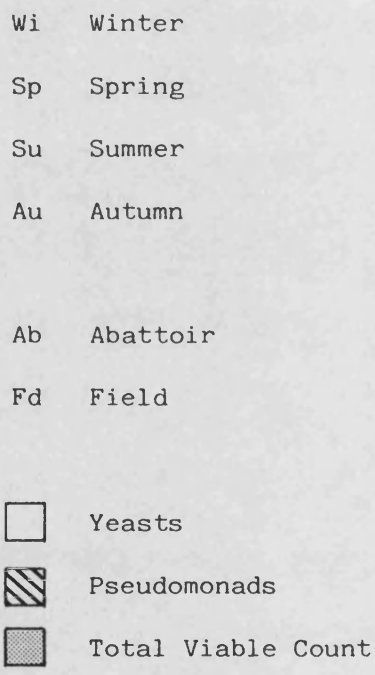
A. incubated in the dark at 25°C for 5 d

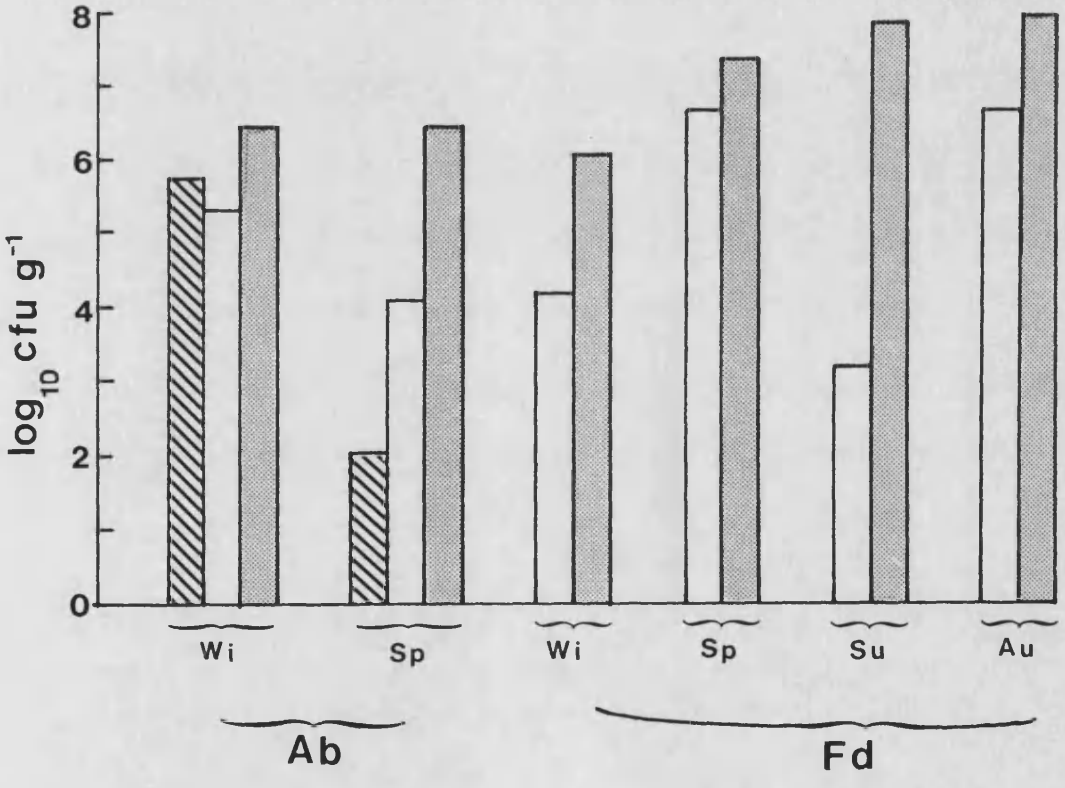
B. continually illuminated (28 μmol $m^{-2}s^{-1}$) at 25°C for 5 d

R - *Rhodotorula* sp.


R
A
B

Figure 10. The recovery of microorganisms from fleece samples:




8
6
4
2
0
log10 cfu g-1
Wi
Sp
Wi
Sp
Su
Au
Ab
Fd

g^{-1} wet wt) of the total viable count (7.41×10^7 g^{-1} wet wt) in July.

Non-pigmented yeasts were the major contaminants of fleece, the pigmented ones accounting for only 5% of the population from January to July and 40% in October (Fig. 8a). The yeasts isolated from fleece were identified with Candida, Cryptococcus and Rhodotorula spp. (Table 19).

Yeasts accounted for 6.9% and pseudomonads 18.8% of the total microflora of the fleece samples taken in the slaughterhouse in Winter. The proportions of both were reduced in samples taken in Spring. Alcaligenes and Flavobacterium spp. were the dominant organisms on Plate Count Agar.

c) In the Slaughterhouse

One municipal slaughterhouse with the same team of workers was surveyed throughout the year. The working practices in the slaughterhouse precluded the sampling of known areas of carcasses. Swabs were taken from the carcass surface without using a standardised grid. As the emphasis was given to the ratio of yeasts to bacteria, this was not considered to be of major importance simply because the yeast population and the total viable count originated from just the same sample site on every occasion. Thus the back, belly and tail of the carcass were sampled after defleecing and after washing (Figs. 11, 13). The wash water dripping off the carcass and the butchers' aprons were also sampled routinely (Figs. 11, 12).

Figure 11. The recovery of microorganisms from lambs slaughtered in Winter.

Average of 5 samples

Symbols - see below

Figure 12. The recovery of microorganisms from slaughtermens' aprons.

B Belly

DW Drip water (ml^{-1})

AD After defleecing

AW After washing

AE At evisceration

Wi Winter

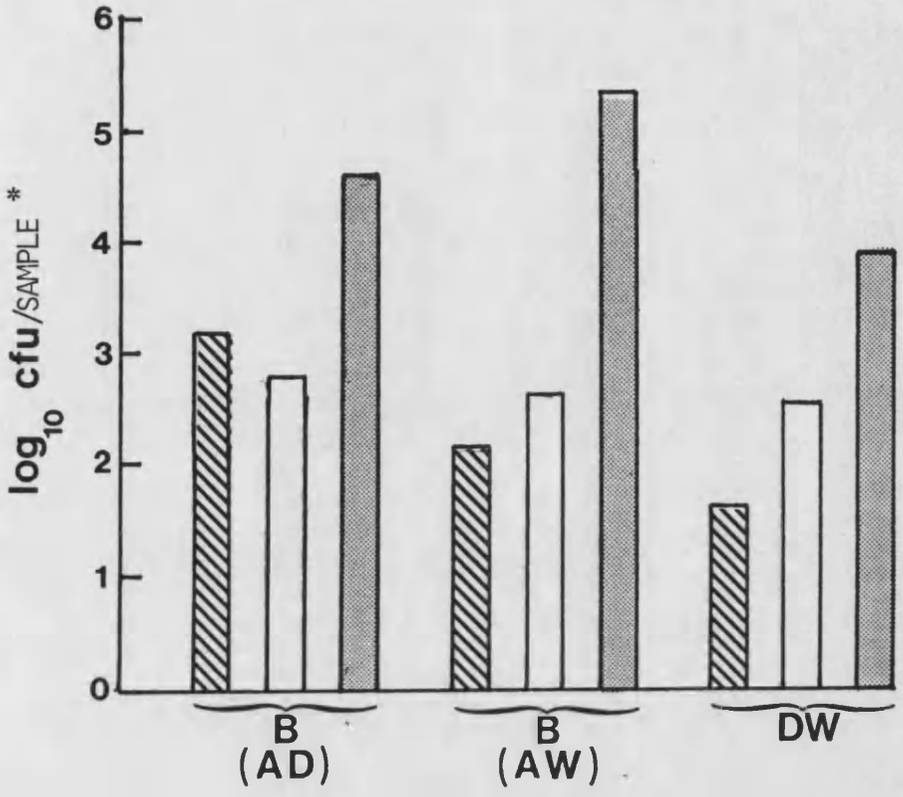
Sp Spring

Pseudomonads

Yeasts

Total Viable Count

* details in text


log10 cfu/SAMPLE *
6
5
4
3
2
1
0
B
(AD)
B
(AW)
DW

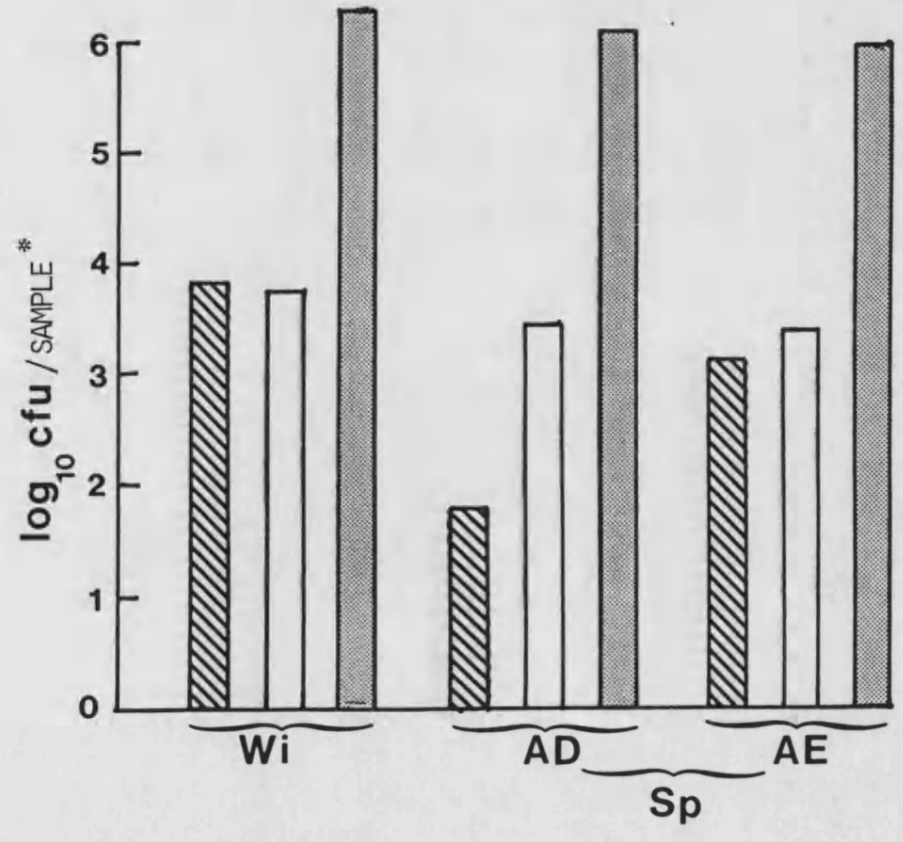

log10 cfu / SAMPLE *
6
5
4
3
2
1
0
Wi
AD
AE
Sp

Figure 13. The recovery of microorganisms from lamb carcasses:

a) After defleecing - Spring

b) After washing - Spring

c) In the Summer

d) In the Autumn

Ba	Back	N	Neck
B	Belly	S	Slit
T	Tail	DW	Drip water (ml^{-1})
AD	After defleecing	AW	After washing

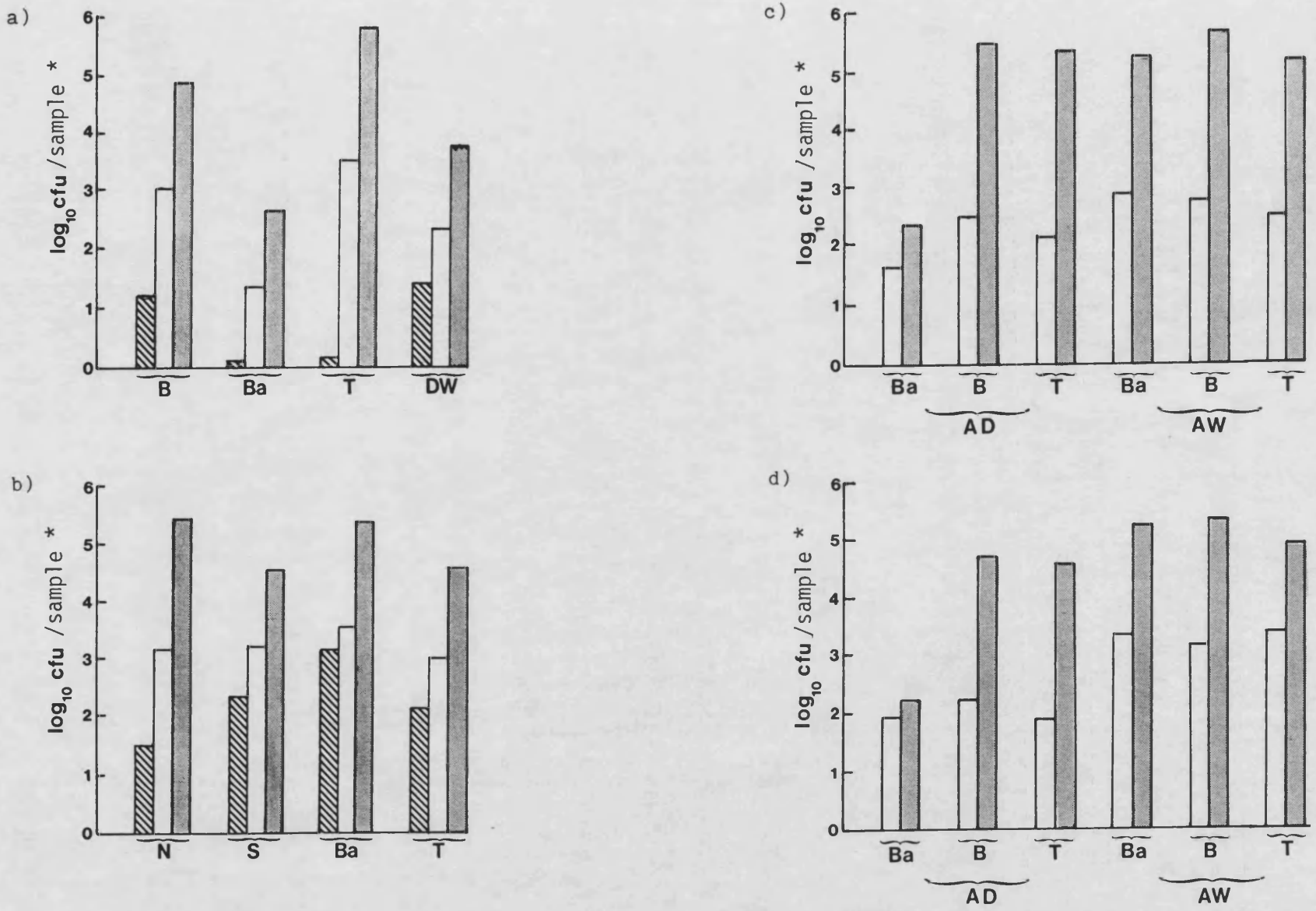
Pseudomonads

Yeasts

Total Viable Count

Average of 3-6 samples

* details in text


a)
log10 cfu /sample *
0
1
2
3
4
5
6
B
Ba
T
DW
b)
log10 cfu /sample *
N
S
Ba
T
c)
log10 cfu /sample *
Ba
B
T
Ba
B
T
AD
AW
d)
log10 cfu /sample *
Ba
B
T
Ba
B
T
AD
AW

Analysis of the microflora recovered by sampling the carcass surface throughout the year (Figs. 11, 13) again revealed that the number of yeasts (9.89×10^1 to 3.0×10^3) were far fewer (0.057 - 5.00%) than those of bacteria (Table 18b) as indicated by the total viable count (3.7×10^4 to 4.2×10^5). The only exception to this was the back region of the carcass after defleecing. In this instance few yeasts ($0.25 - 1.0 \times 10^2$) were recovered in association with an exceptionally low count of bacteria ($1.5 - 5.0 \times 10^2$). The microbial ratio on the back of the carcass, however, was altered subsequently as a consequence of the carcass being washed with a cold water hose. Yeasts accounted for only 0.5 - 1.3% ($0.85 - 2.6 \times 10^3$) of the increased total viable count ($1.7 - 2.3 \times 10^5$). Washing did not change the ratio of yeasts vis à vis bacteria on the belly or tail region of the carcass.

The non-pigmented yeasts (the dominant yeasts) were identified with Candida and Cryptococcus spp. (Table 19). The few pigmented yeasts isolated from the carcass surface were identified with Rhodotorula spp. (Table 19). Representatives of all three genera were isolated from the rubberised aprons worn by the butchers who manually removed the fleece of sheep supported initially on a cradle and subsequently from an overhead rail. it is noteworthy that the genera of yeasts isolated in this study from carcasses and the slaughterhouse environment were the same as those recovered from similar situations by Baxter and Illston (1976, 1977), Dalton (1984) and Lowry (1984).

Pseudomonads (on CFC medium) accounted for only a small percentage (0.014 - 4.05%) of the microflora on the carcass surface

in Winter and Spring (Figs. 11, 13a, b) as indicated by the total viable count on Plate Count Agar. Alcaligenes and Flavobacterium spp. were dominant on the latter. Kelly (1978) noted that 14% of the initial microflora (isolated at 25°C) of lamb carcasses were Flavobacterium spp. whereas pseudomonads accounted for only 9.5%.

d) In Lamb Products

Yeasts were only a minor (0.05 - 2.0%) component vis à vis the total viable count of unsulphited minced lamb. Indeed the percentage range was comparable to that found in the field samples, the fleece and the carcass. The relative contribution of yeasts to contamination tended to diminish with storage of minced lamb at 5°C (Table 20).

Sulphite addition (500 µg SO_2 g^{-1}) to minced lamb from a local butcher's shop was associated with extensive growth of yeasts such that after 4 d storage at 5°C these organisms attained numbers (7.7×10^6 g^{-1} meat) greater (179.7%) than those of the total bacterial population (4.3×10^6 g^{-1} meat) - Table 20. Minced lamb obtained from a supermarket contained a high bacterial count before the addition of sulphite. In this instance yeasts did increase as a ratio of the total contamination but they did not dominate the microflora during 6 d storage at 5°C. Although these observations confirm those of others (Banks, 1983; Dalton, 1984), namely that sulphite selects yeasts in meat products, they indicate also that the eventual size of the yeast population in sulphited minced lamb is determined by an interplay of factors, one of which is obviously competition between yeasts and the bacteria which would normally

Table 20. Yeasts as a percentage of Total Viable Count.

A) Minced lamb - butchers stored at 5°C

	DAYS						
	0	1	2	3	4	5	7	10
Control	2.0	0.5	0.1	0.1	0.01	0.002		
Sulphited	2.0	21.0	45.6	15.0	179.7	116.0	341.9	65.5

B) Minced lamb - supermarket stored at 5°C

	DAYS						
	0	1	2	3	4	5	6
Control	0.05	0.14	0.08	0.04	0.02	0.02	0.01
Sulphited	0.05	0.34	0.43	0.43	4.30	6.32	29.40

C) Lamb burgers - sulphited

	DAYS									
	0	1	2	3	6	8	10	14	17	24
5°C	1.88	1.42	5.60	136.08	76.72	99.20	74.83	56.01	38.11	38.46
15°C	1.88	25.54	126.67	99.88	4.67	9.64	20.68	42.76		

dominate the microbial association of minced meat.

A situation similar to that described above was noted with sulphited lamb burgers stored at 5°C and 15°C (Table 20). The yeast flora accounted for 126.67% after 2 d storage at 15°C and 136% after 3 d at 5°C, as compared to the total viable counts of bacteria. It was noted, however, that the dominance of yeasts was not a permanent feature. As will be described in greater detail subsequently, renewed growth of bacteria towards the end of storage was associated with loss of sulphite through binding with products of yeast metabolism.

It is evident from these observations that sulphite favours the growth of yeasts in lamb products. There was no evidence, however, of sulphite selectively favouring a particular genus. Indeed, those (Candida, Cryptococcus and Rhodotorula spp.) found in the pastures and abattoir were also the principal organisms in unsulphited and sulphited minced lamb and lamb products (Table 21).

2. The Microbial Association in Minced Lamb Products

It was evident from the literature review (Section 5) of sulphite preservation of meat products - mainly the British fresh sausage - that sulphite selected a microbial association of Gram-positive bacteria (Brochothrix thermosphacta and homofermentative lactobacilli) and yeasts (Brown, 1977; Banks, 1983; Dalton, 1984). The present study was undertaken, therefore, to confirm whether or not the previous observations with sulphited sausages applied to sulphited lamb products.

Table 21. Yeast species isolated from minced lamb and lamb products.

Species
Candida famata
Candida humicola
Candida inconspicua
Candida lipolytica
Candida mesenterica
Candida norvegica
Candida sake
Candida vini
Candida zeylanoides
Cryptococcus albidus var. *albidus*
Cryptococcus infirmo-minatus
Cryptococcus laurentii
Rhodotorula minuta
Rhodotorula rubra
*Trichosporon cutaneum**

* Only isolated from unsulphited minced lamb obtained from a supermarket

Sodium metabisulphite was added to minced lamb to give a final concentration of ca 500 μg SO_2 g^{-1}. The presence of sulphite in minced lamb stored at 5°C reduced the rate and extent of growth of the bacteria (total viable count) recovered on Plate Count Agar. When minced lamb was lightly contaminated, as with that obtained from a butcher's shop, sulphite prevented bacterial growth for upwards of two days and extended the shelf-life by ca 3 d (Fig. 14b). With the heavily contaminated minced lamb obtained from a supermarket, the growth of bacteria was inhibited throughout storage (Fig. 14a). This feature was not associated with an extension in shelf-life. These observations were in accord with those noted with minced beef and pork (Nychas, 1984) and with British fresh sausage (Banks, 1983; Dalton, 1984). Judging from the results obtained with sulphited lamb burgers (supplemented with rusk, soya flour and starch), storage at 5° was more effective than 15°C in suppressing the growth of the general bacterial counts. At the latter temperature, an increase in total viable count accompanied the rapid loss of free sulphite (Figs. 21b, 24b).

About equal numbers of pseudomonads and Br. thermosphacta were present in minced lamb at the time of purchase. The former (isolated on CFC medium - Mead and Adams, 1977) became dominant in unsulphited mince with storage at 5°C for 5 - 6 d (Figs. 14, c, d). They did not grow during 5 d storage at 5°C in sulphited minced lamb from a supermarket (Fig. 14c). Growth of pseudomonads occurred, however, when the free sulphite concentration of minced lamb obtained from a butcher's shop fell below a critical level (160 μg g^{-1}) with 10 d storage at 5°C (Figs. 14d, 20b). Even so the

Figure 14. The effect of sulphite in minced lamb stored at 5°C on the:

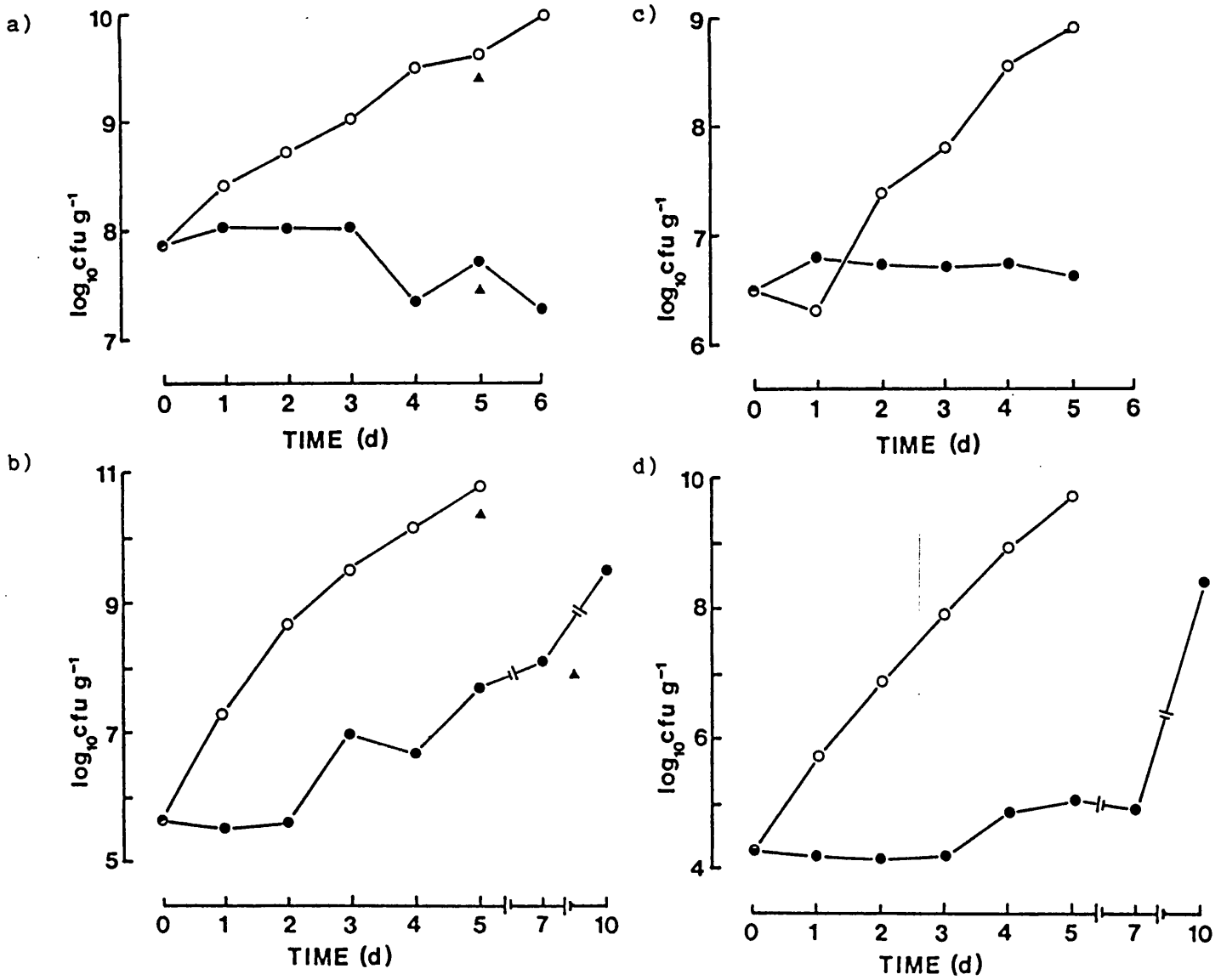
Total Viable Count (recovered on PCA)
a) Supermarket
b) Butcher's shop

Pseudomonads (recovered on CFC medium)
c) Supermarket
d) Butcher's shop

○ Unsulphited ● Sulphited

▲ Detectable spoilage

Points - mean of 3 observations.


a)
b)
c)
d)
$\log_{10}$cfu g^{-1}
TIME (d)
0 1 2 3 4 5 6
0 1 2 3 4 5 7 10

size of the climax population of pseudomonads at the time of spoilage was always larger in unsulphited than sulphited minced lamb (Figs. 14c, d).

The growth of pseudomonads in lamb burgers was markedly impeded with storage at 5°C but not at 15°C (Fig. 21d). The amount of free sulphite diminished rapidly at 15°C and growth stasis of these organisms was released once the critical threshold value of the preservative was reached by 7 d (Fig. 24 b).

Enterobacteriaceae, the most sulphite sensitive contaminants, were a minor component of the initial microflora of minced lamb. Numbers of Enterobacteriaceae recovered on VRBA and VRBG did not increase with storage of sulphited minced lamb from a supermarket at 5°C for 6 d (Figs. 15a, c). The numbers of these organisms eventually increased in minced lamb obtained from a butcher's shop but only after 10 d storage at which time the free sulphite concentration was ca 50 μg g^{-1} (Figs. 15b, d, 20b). The delayed onset of growth was associated with climax populations smaller than those obtained in unsulphited meat (Figs. 15b, d). The marked sensitivity of Enterobacteriaceae vis à vis pseudomonads to sulphite was emphasised by comparison of generation times (Figs. 18a, b, 23). This also confirmed that Enterobacteriaceae in lamb burgers were more sensitive to sulphite inhibition with storage at 5°C than at 15°C (Figs. 22a, b, 24b, c).

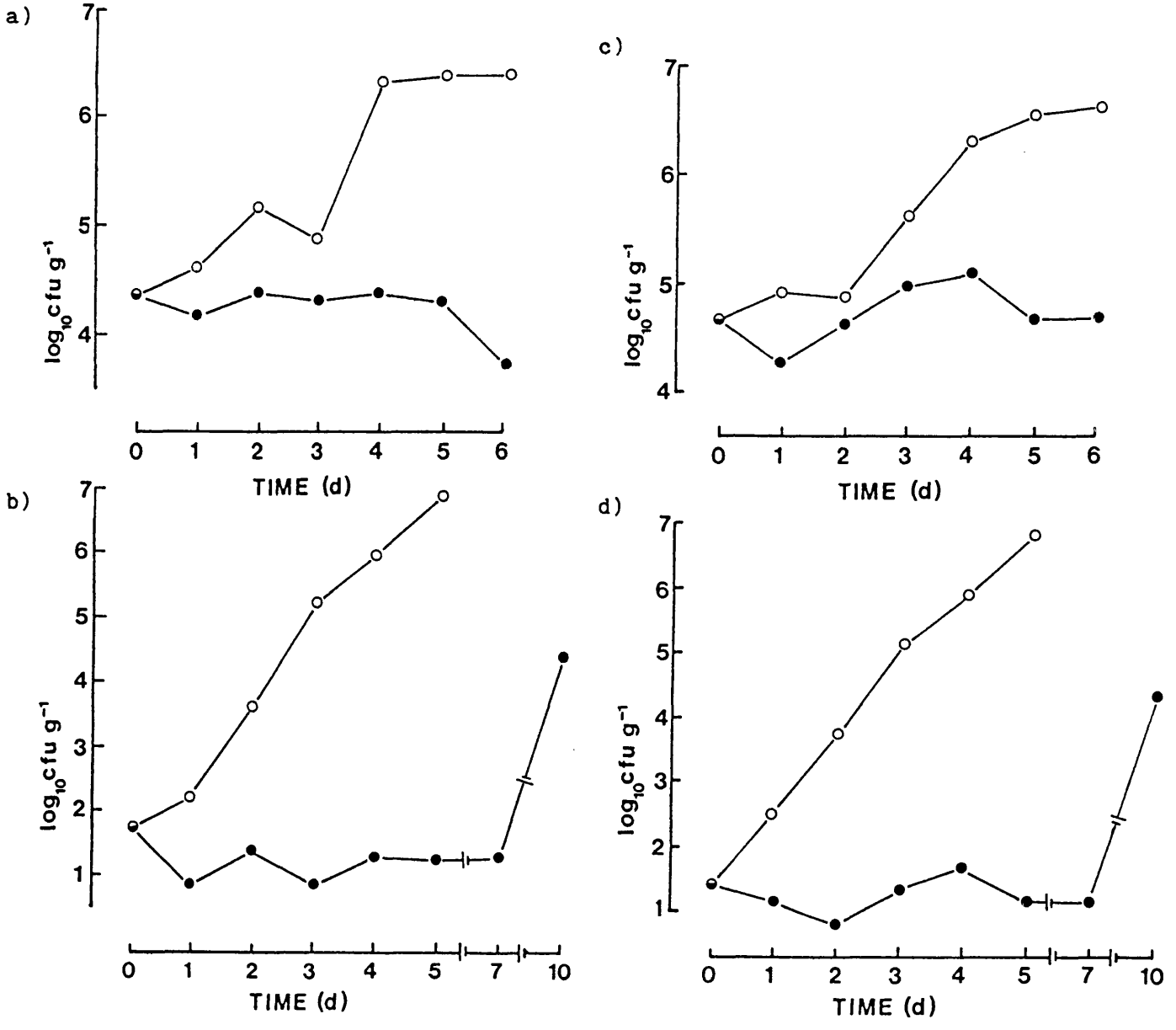
Growth of Enterobacteriaceae occurred, however, when the free sulphite level fell below the critical threshold value (40 μg SO_2 g^{-1}) as noted after 6-8 d storage at 15°C with lamb burgers (Figs. 22a, b, 24b).

Figure 15. The effect of sulphite in minced lamb stored at 5°C on the growth of Enterobacteriaceae:

Recovered on VRBA
a) Supermarket
b) Butcher's shop

Recovered on VRBG
c) Supermarket
d) Butcher's shop

○ Unsulphited ● Sulphited


a)
b)
c)
d)
log10cfu g-1
TIME (d)
0 1 2 3 4 5 6
0 1 2 3 4 5 7 10

Although showing much less sensitivity to sulphite than the organisms considered so far, Br. thermosphacta (isolated on STAA medium - Gardner, 1966) formed a major component of the microflora of both unsulphited and sulphited minced lamb after 5-6 d storage at 5°C (Figs. 16a, b). Sulphite had little effect on the rate or extent of growth of Br. thermosphacta (Fig. 16b) in minced lamb obtained from a butcher's shop. The presence of sulphite, however, retarded the growth rate and curtailed the size of the climax population of this organism in minced lamb from a supermarket (Fig. 18a). Brochothrix thermosphacta also persisted as one of the dominant organisms in the microbial association of sulphited lamb burgers at 5°C for 14 d and 15°C for 5 d (Fig. 21c).

Lactobacilli (isolated on lactobacilli selective medium - Keddie, 1951) formed a major component of the microflora of sulphited minced lamb. The presence of the preservative had little effect on the rate or extent of growth of these organisms (Figs. 16c, d, 18a, b), an observation in agreement with that noted in minced beef by Nychas (1984). The growth rate and the size of the climax population of lactobacilli in sulphited lamb burgers was unaffected by temperature, both being similar at 5°C and 15°C (Figs. 22c, 23).

Enterococci were recovered on Kanamycin Aesculin Azide agar. These organisms accounted for only a minor part of the microflora in unsulphited or sulphited minced lamb and lamb burgers. Sulphite had little effect on the rate or extent of growth of Enterococci in minced lamb at 5°C (Figs. 17a, b, 18a, b), a feature noted in sausages by Banks (1983). The growth of these organisms in

Figure 16. The effect of sulphite in minced lamb stored at 5°C on the growth of:

Brochothrix thermosphacta (recovered on STAA)	a) Supermarket b) Butcher's shop
lactobacilli (recovered on Keddie (1951) medium)	c) Supermarket d) Butcher's shop

○ Unsulphited ● Sulphited

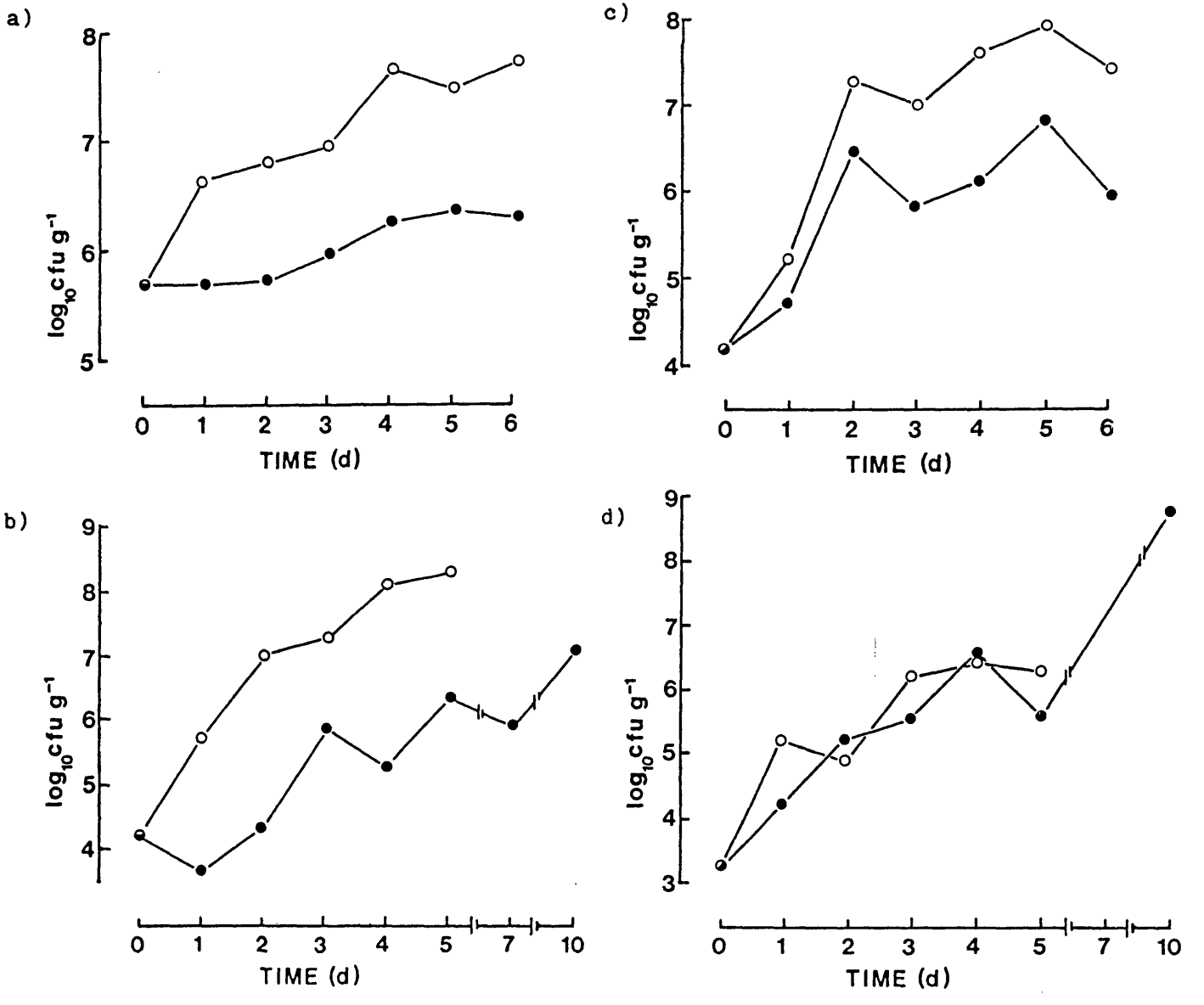

a)
b)
c)
d)
log10cfu g-1
TIME (d)
0 1 2 3 4 5 6
0 1 2 3 4 5 7 10

Figure 17. The effect of sulphite in minced lamb stored at 5°C on the growth of:

Enterococci (recovered on Kanamycin aesculin azide agar)
a) Supermarket
b) Butcher's shop

Yeasts (recovered on RBC)
c) Supermarket
d) Butcher's shop

○ Unsulphited ● Sulphited

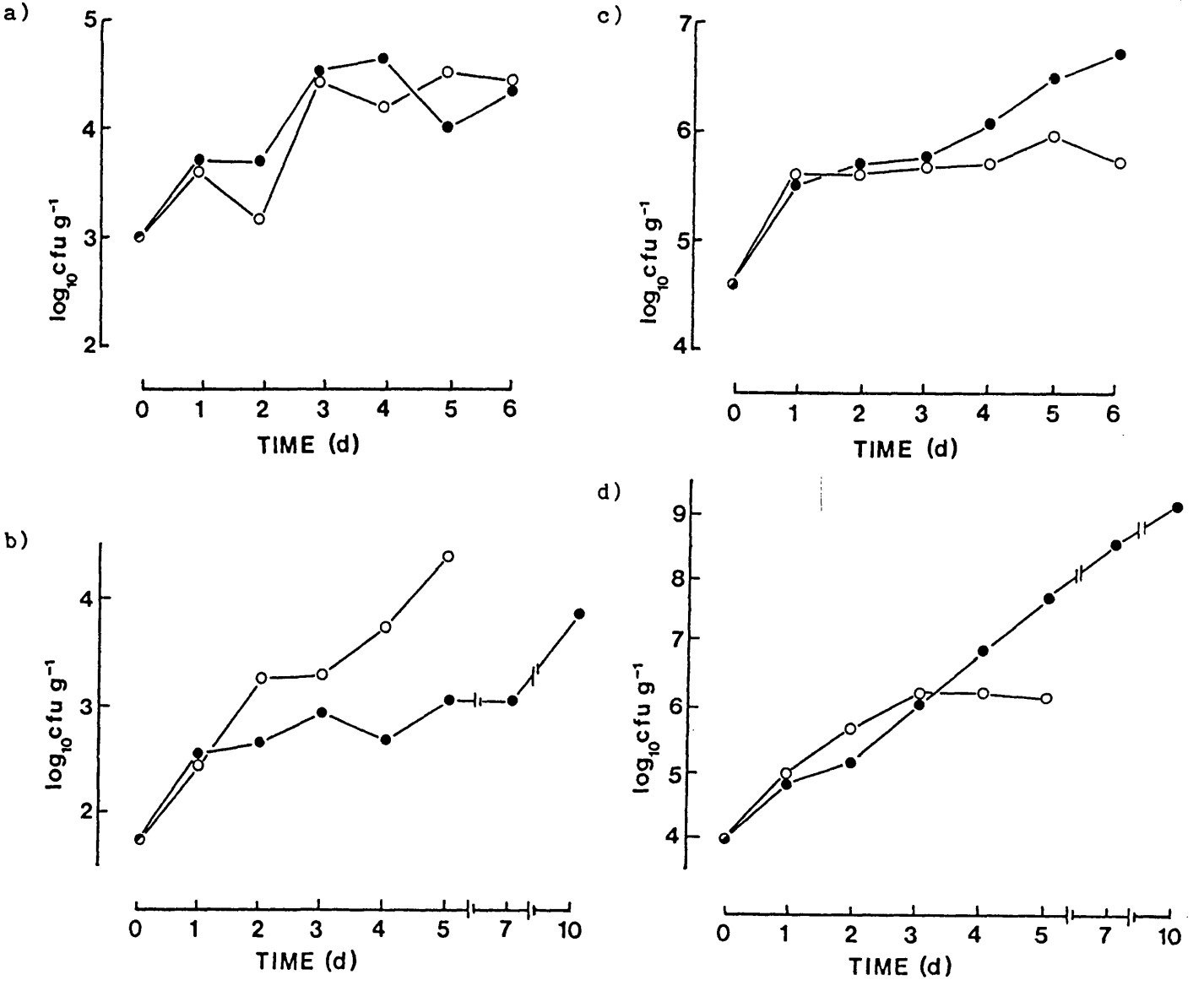

a)
b)
c)
d)
log10cfu g-1
TIME (d)
0 1 2 3 4 5 6
0 1 2 3 4 5 7 10

Figure 18. The effect of sulphite on the rate (mean doubling time) and extent of growth (climax population) of microorganisms in minced lamb stored at 5°C, obtained from:

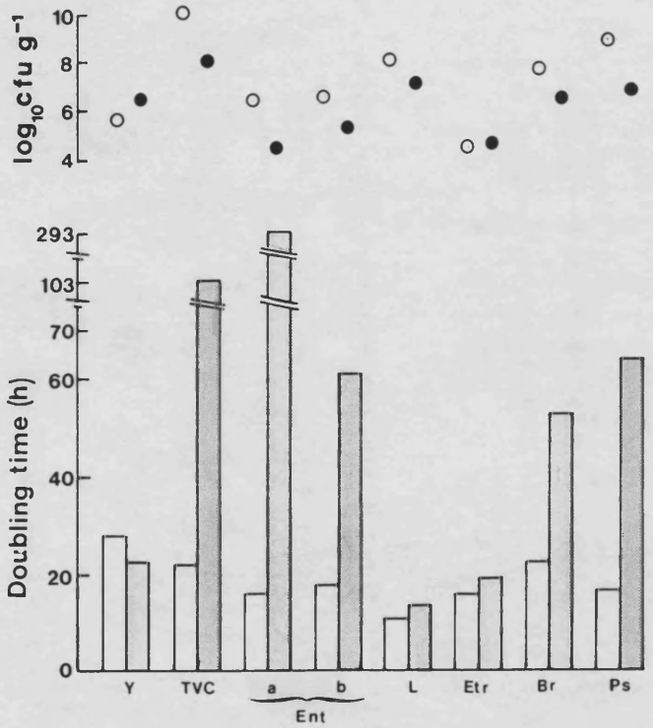
a) Supermarket

b) Butcher's shop

 ○ Unsulphited  ● Sulphited

Y	Yeasts	
TVC	Total Viable Count	
Ent	Enterobacteriaceae	a) recovered on VRBA
		b) recovered on VRBA
L	Lactobacilli	
Etr	Enterococci	
Br	Brochothrix thermosphacta	
Ps	Pseudomonads	

a)


log10cfu g-1
10
8
6
4
293
103
70
60
40
20
0
Doubling time (h)
Y
TVC
a
b
Ent
L
Etr
Br
Ps

b)

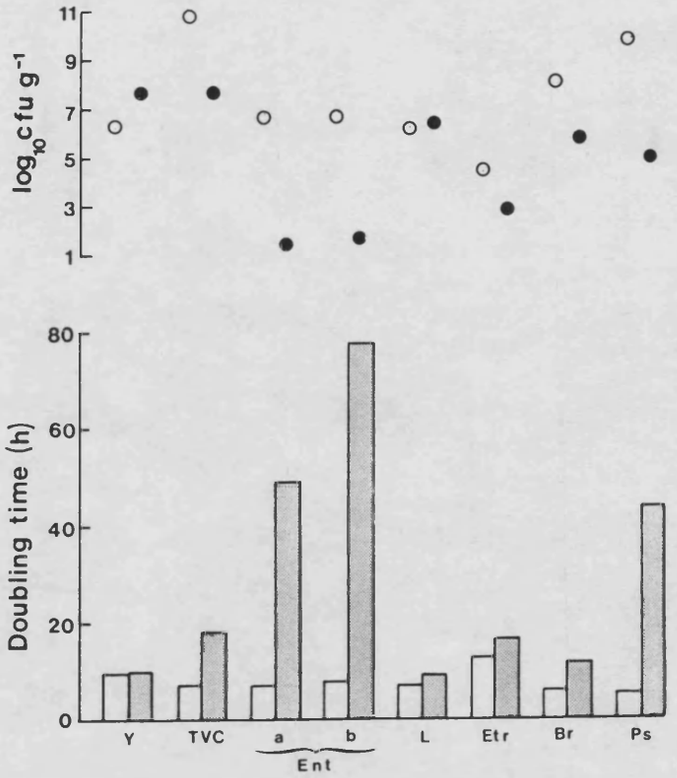

log10cfu g-1
11
9
7
5
3
1
80
60
40
20
0
Doubling time (h)
Y
TVC
a
b
Ent
L
Etr
Br
Ps

Figure 19. The percentage of yeasts giving a positive reaction on acetaldehyde detection medium (OGYA + Schiff's reagent) in minced lamb obtained from a butcher's shop and stored at 5°C for 7 d.

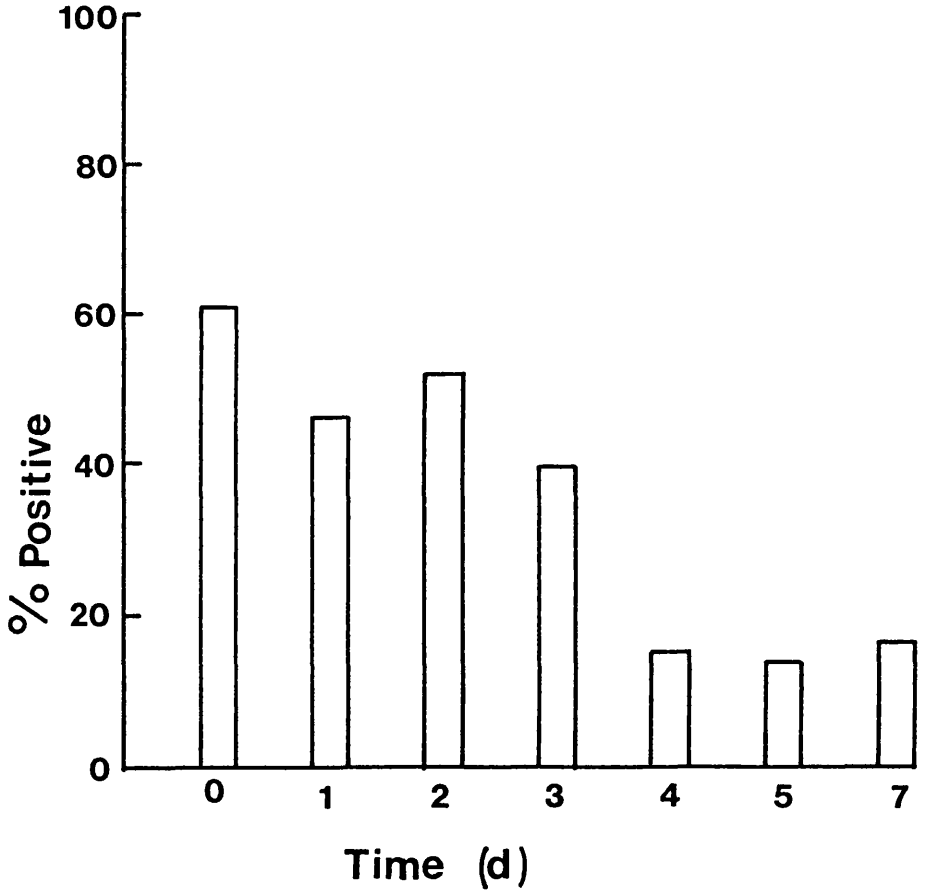

100
80
60
40
20
0
% Positive
0
1
2
3
4
5
7
Time (d)

Figure 20. The concentration of free, bound and total sulphite in minced lamb stored at 5°C obtained from:

a) Supermarket

b) Butcher's shop

■ Bound

□ Free

△ Total

The effect of sulphite on the pH of minced lamb stored at 5°C obtained from:

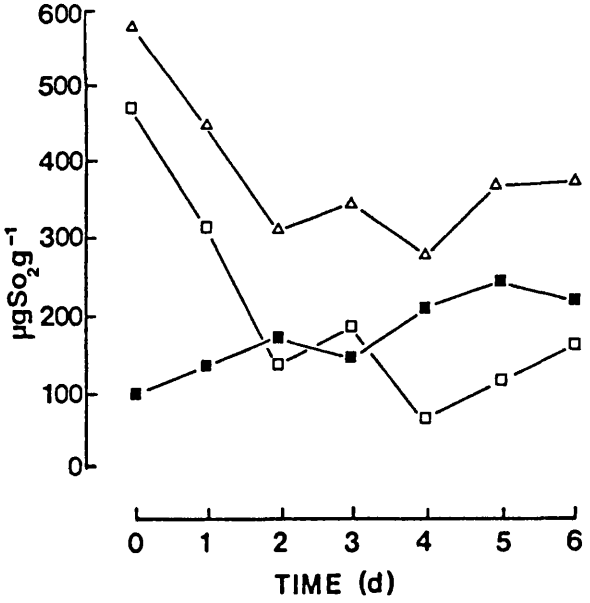
c) Supermarket

d) Butcher's shop

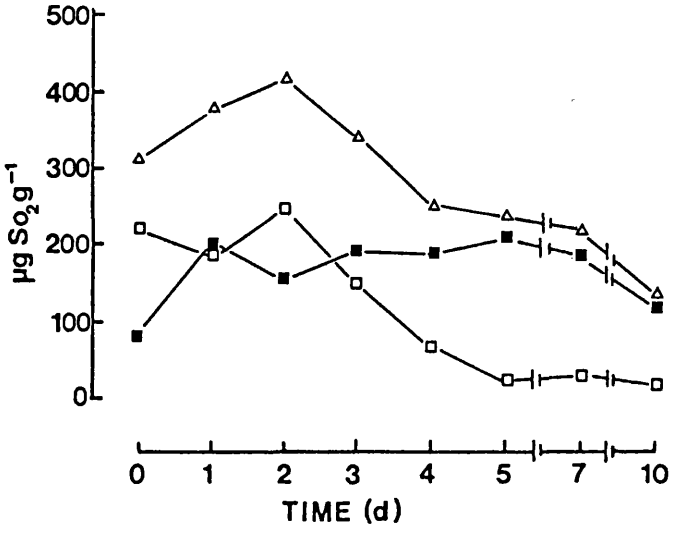
○ Unsulphited

● Sulphited

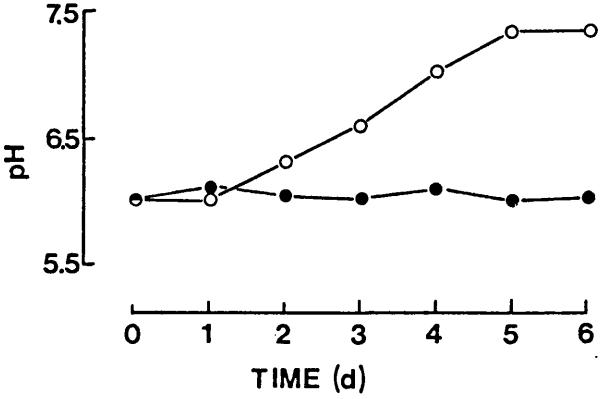
a)


600
500
400
300
200
100
0
µgSo2g-1
0 1 2 3 4 5 6
TIME (d)

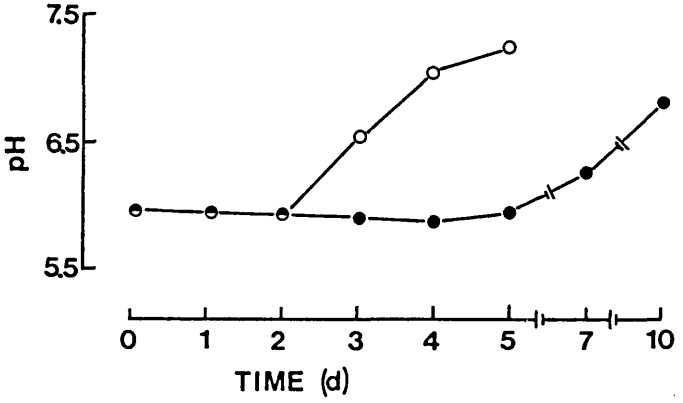
b)


500
400
300
200
100
0
µg So2g-1
0 1 2 3 4 5 7 10
TIME (d)

c)


7.5
6.5
5.5
pH
0 1 2 3 4 5 6
TIME (d)

d)


7.5
6.5
5.5
pH
0 1 2 3 4 5 7 10
TIME (d)

sulphited lamb burgers was influenced by temperature. The growth was inhibited at 5°C, whereas a relatively rapid growth rate was observed at 15°C (Figs. 22d, 23).

Yeasts (isolated on Rose Bengal chloramphenicol agar) were only a minor component of the initial contamination of minced lamb. As noted previously (Table 20a) in the presence of sulphite, however, these organisms became numerically dominant after 4 d storage at 5°C in what had been lightly contaminated minced lamb at the time of purchase (Fig. 17d). Sulphite had no effect on the rate of growth of yeasts in minced lamb obtained from a butcher's shop (Fig. 18b). In contrast, the preservative, caused a reduction in generation time of these organisms in minced lamb from a supermarket noted for its heavy contamination at the time of purchase (Figs. 17c, 18a). Additional evidence of yeast proliferation in the presence of sulphite came from studies of sulphited lamb toppers, lamb burgers and lamb with mint sausages (Fig. 25). In most of these sulphited products the size of the climax population of yeasts after 6 d storage at 5°C was 10^6 - 10^7 c.f.u.g^{-1} meat as compared to 10^4 - 10^5 c.f.u.g^{-1} meat in unsulphited products such as lamb en croute and minced lamb (Fig. 25). Yeasts, lactobacilli and Br. thermosphacta in about equal numbers, dominated the microflora of sulphited lamb burgers after 14 d storage at 5°C (Fig. 21a, c, 22c). As the numbers of Br. thermosphacta were reduced after 8 d storage (Fig. 21c) yeasts and lactobacilli dominated after 14 d at 15°C.

Candida spp. (73% of isolates), Cryptococcus spp. (21%) and Rhodotorula spp. (6%) were the common and dominant contaminants of

Figure 21. The effect of temperature of storage on the growth of microorganisms in sulphited lamb burgers:

a) Yeasts

b) Total Viable Count

c) Brochothrix thermosphacta

d) Pseudomonads

● 5°C

○ 15°C

▲ Detectable spoilage

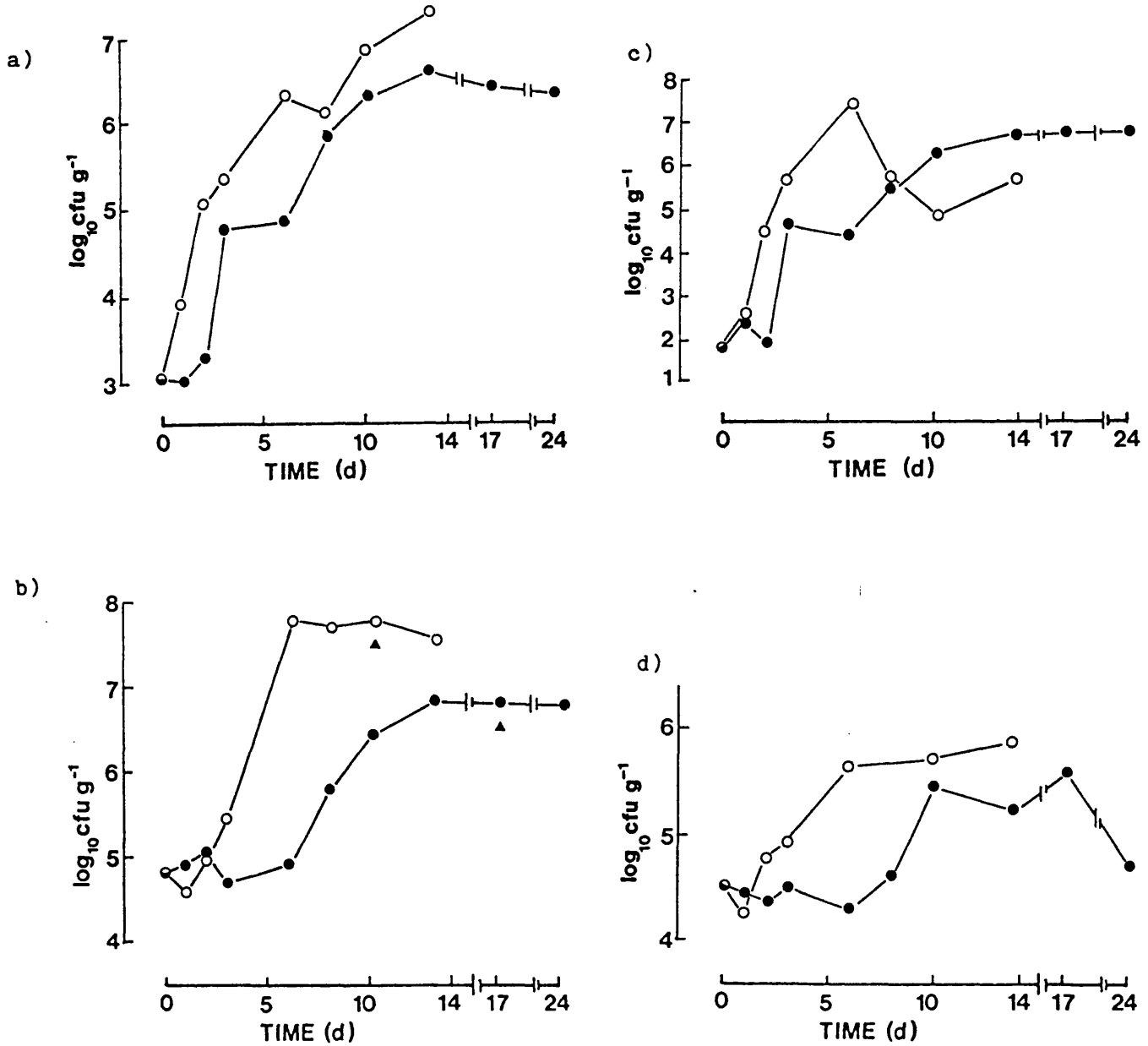

a)
b)
c)
d)
log10 cfu g-1
TIME (d)
0
5
10
14
17
24

Figure 22. The effect of temperature of storage on the growth of bacteria in sulphited lamb burgers:

a) Enterobacteriaceae (on VRBG)

b) Enterobacteriaceae (on VRBA)

c) Lactobacilli

d) Enterococci

● 5°C

○ 15°C

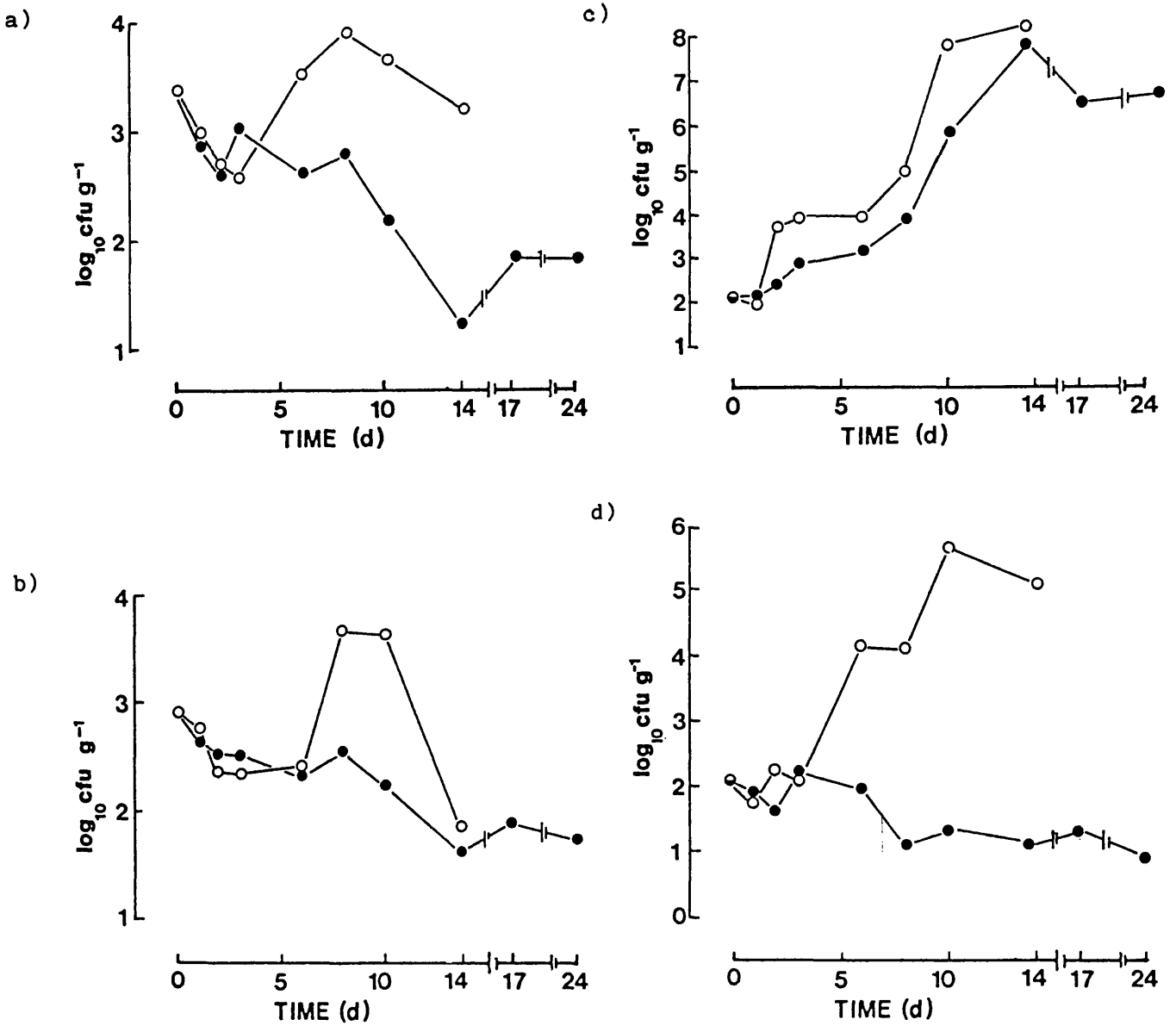
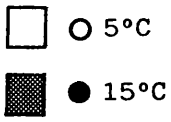

a)
b)
c)
d)
log10 cfu g-1
TIME (d)
0
5
10
14
17
24

Figure 23. The effect of temperature of storage on the rate (mean doubling time) and extent (climax population) of microorganisms in sulphited lamb burgers.

Y	Yeasts	
TVC	Total Viable Count	
Ent	Enterobacteriaceae	a) on VRBA
		b) on VRBG
L	Lactobacilli	
Etr	Enterococci	
Br	Brochothrix thermosphacta	
Ps	Pseudomonads	



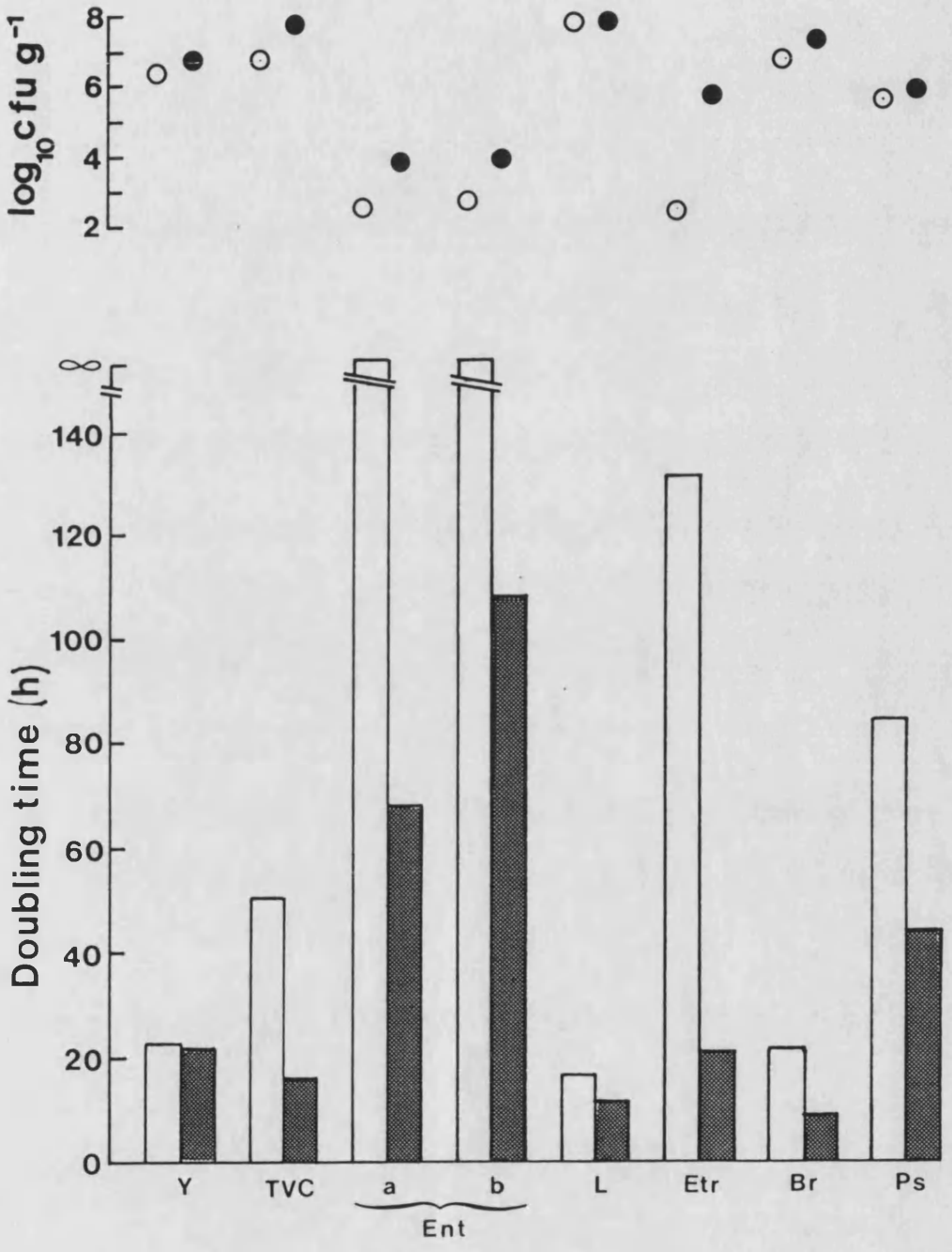

log10 cfu g-1
8
6
4
2
Doubling time (h)
∞
140
120
100
80
60
40
20
0
Y
TVC
a
b
Ent
L
Etr
Br
Ps

Figure 24. The effect of the temperature of storage of lamb burgers on:

a) pH

● 5°C ○ 15°C

The concentration of free, bound and total sulphite:

b) 15°C

c) 5°C

□ Free △ Total

■ Bound

d) total sulphite:

△ 5°C DTNB Assay

▲ 15°C DTNB Assay

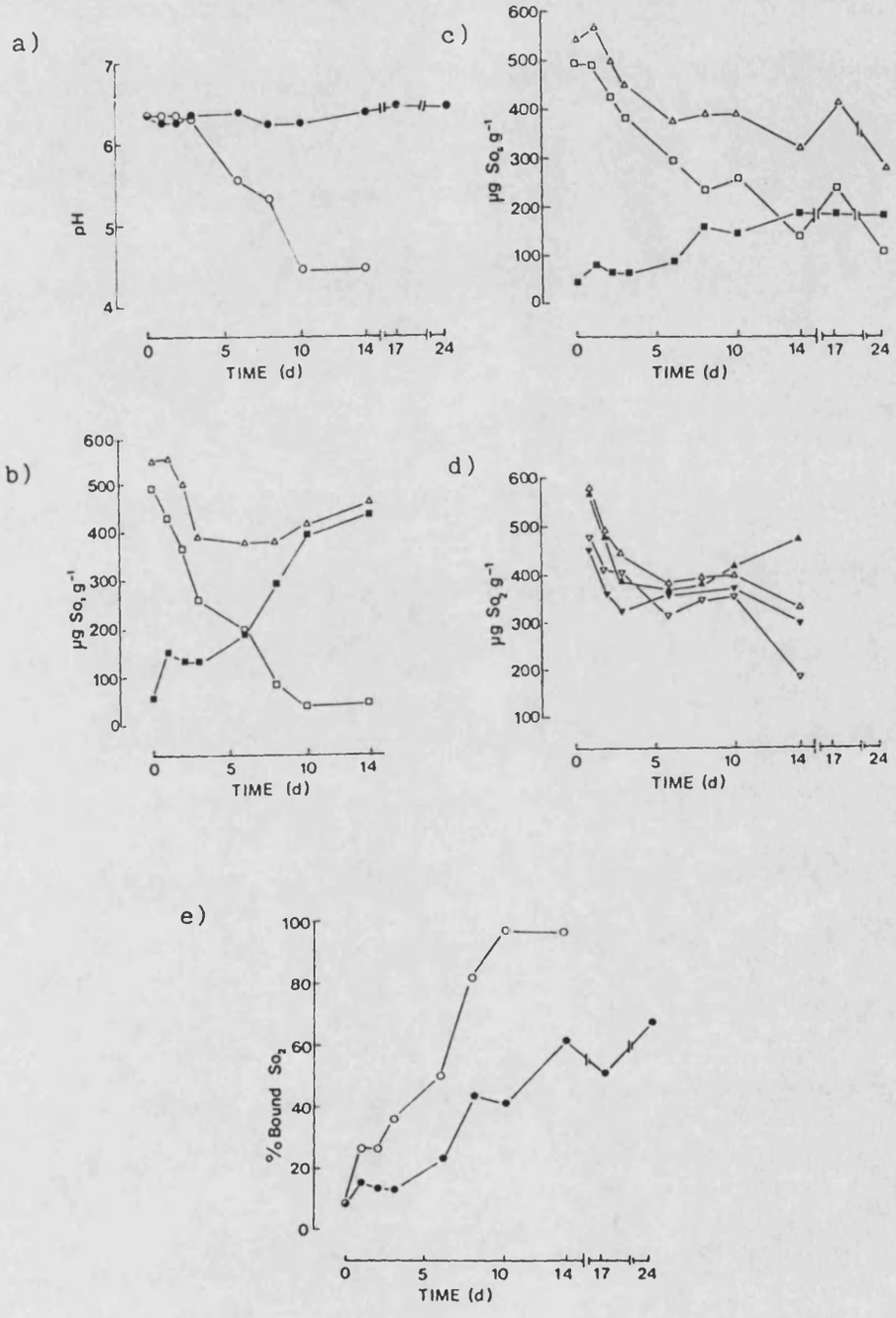
▽ 5°C Boehringer Mannheim Assay

▼ 15°C Boehringer Mannheim Assay

e) The amount of bound sulphite as a percentage of total sulphite :

● 5°C

○ 15°C


a)
pH
7
6
5
4
0 5 10 14 17 24
TIME (d)
c)
µg So2 g-1
600
500
400
300
200
100
0
0 5 10 14 17 24
TIME (d)
b)
µg So2 g-1
600
500
400
300
200
100
0
0 5 10 14
TIME (d)
d)
µg So2 g-1
600
500
400
300
200
100
0 5 10 14 17 24
TIME (d)
e)
% Bound So2
100
80
60
40
20
0
0 5 10 14 17 24
TIME (d)

minced lamb and lamb products (Table 21). This observation is in agreement with that of Nychas (1984) who found that 60% of the yeasts isolated from beef were Candida spp., 10% Cryptococcus spp. and 3% Rhodotorula spp. Additionally he isolated strains of Trichosporon, Debaryomyces and Pichia. Hseih and Jay (1984) noted that 82% of the yeast flora recovered from beef comprised Candida spp. and that Trichosporon spp. and Cryptococcus spp. were only a minor part of the flora. Indeed, Trichosporon cuteaneum was only isolated from unsulphited minced lamb obtained from a supermarket (Table 21).

The rapid growth of pseudomonads in unsulphited minced lamb was accompanied by an alkaline drift in pH. In sulphited minced lamb the pH (5.8 - 6.0) did not change for 5-6 d (Figs. 20c, d) and an alkaline drift was only observed in minced lamb obtained from a butcher's shop after 10 d storage at 5°C (Fig. 20d). Similarly, the pH of various sulphited lamb products (Figs. 26a, b) stored at 5°C did not change. Indeed the pH (ca 6.2) of lamb burgers was unchanged over 24 d storage at 5°C (Fig. 24a). At 15°C the growth of lactobcilli was associated with an acid drift. Similar trends were noted in sausages by Banks (1983). The growth of Br. thermosphacta in lamb burgers at 15°C was arrested after 8 d storage when the pH diminished to 5.25 and Enterobacteriaceae were inhibited when the pH declined to 4.4 (Fig. 24a).

Sodium metabisulphite added to minced lamb to give a final concentration of ca 500 μg SO_2 g^{-1} conserved the red colouration of the meat stored for 5-6 d at 5°C (Plate 2). The concentration of total sulphite, however, in minced lamb diminished rapidly, such

Figure 25. The microbial association in lamb products.

a) Sulphited lamb burgers (A)

▲ Total Viable Count 5°C

△ Total Viable Count 15°C

● Yeasts 5°C

○ Yeasts 15°C

b) Sulphited lamb burgers (B) stored at 5°C

▲ Total Viable Count

△ Yeasts

Sulphited lamb burgers (C)

○ 15°C

● 5°C

c) Yeasts

▼ Sulphited lamb and mint sausages 5°C

○ Minced lamb 5°C

■ Sulphited lamb toppers (with pastry cover) 5°C

□ lamb en croute (with pastry cover) 5°C

d) Yeasts

△ Sulphited lamb toppers 5°C

● English minced lamb 1°C

○ New Zealand minced lamb 1°C

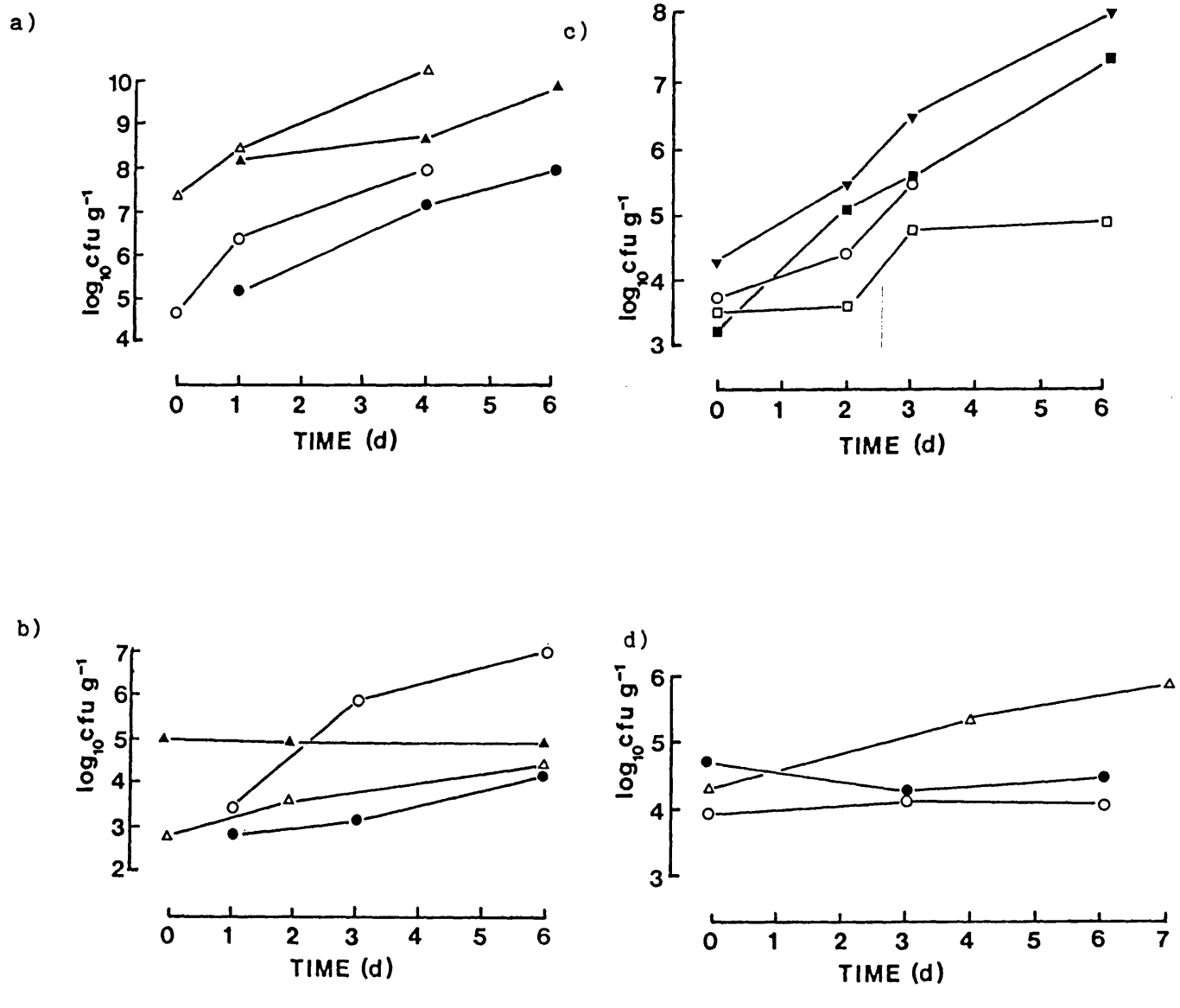

a)
c)
b)
d)
log10cfu g-1
TIME (d)

Figure 26. The pH of lamb products:

a) Sulphited lamb burgers

△ (A) 15°C

▲ (A) 5°C

▽ (B) 5°C

b) Lamb products stored at 5°C

▼ Sulphited lamb and mint sausages

○ Minced lamb

■ Sulphited lamb toppers

□ Lamb en croute

The concentration of acetaldehyde, and free, bound and total sulphite in:

c) Lamb burgers (B)

d) Lamb and mint sausages

e) Lamb toppers

△ Total

■ Bound

□ Free

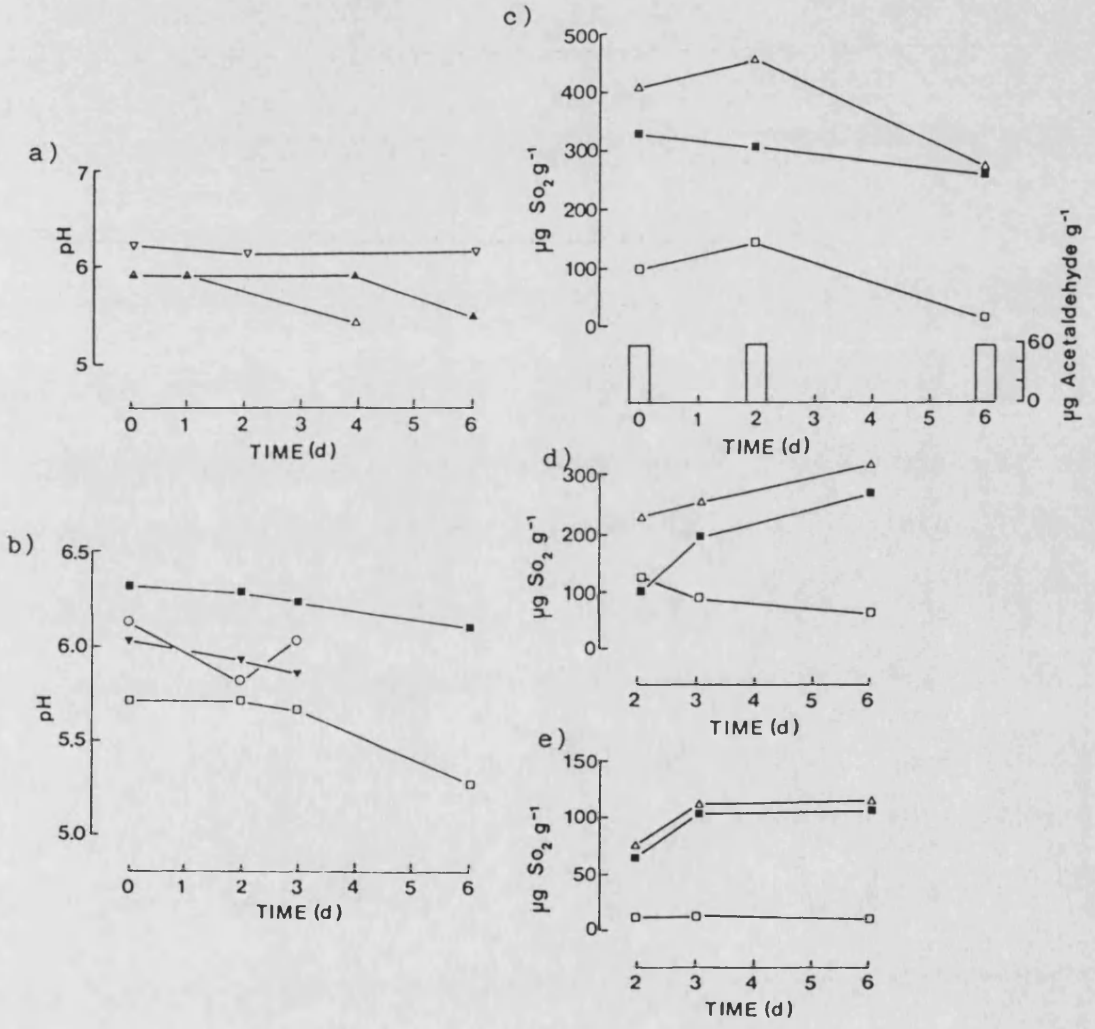

a)
pH
7
6
5
0 1 2 3 4 5 6
TIME (d)
b)
pH
6.5
6.0
5.5
5.0
0 1 2 3 4 5 6
TIME (d)
c)
µg So_2 g^{-1}
500
400
300
200
100
0
60
0
µg Acetaldehyde g^{-1}
0 1 2 3 4 5 6
TIME (d)
d)
µg So_2 g^{-1}
300
200
100
0
2 3 4 5 6
TIME (d)
e)
µg So_2 g^{-1}
150
100
50
0
2 3 4 5 6
TIME (d)

Plate 2. The effect of sulphite on colour retention of minced lamb stored at 5°C for 6 d.

A Unsulphited

B Sulphited (500 μg SO_2 g^{-1})

A B

that only 250 μg g^{-1} (total) remained after 4 d storage at 5°C (Figs. 20a, b). Only 69 μg g^{-1} occurred as free sulphite. With lamb burgers and other minced lamb products (containing additional carbohydrates), total sulphite diminished only slightly during storage at 5°C or 15°C (Figs. 24, 26). Similar trends have been observed with sulphited sausages containing as they do upwards of 12% (w/w) carbohydrates (Banks, 1983; Dalton, 1984). At 15°C there was a rapid loss of free and concomitantly an increase in bound sulphite in lamb burgers. After 10 d storage at 15°C, 93% of the total sulphite was bound whereas only 34% was bound at 5°C (Fig. 24e). A parallel situation was noted in sausages where a greater loss of free sulphite occurred at higher storage temperatures (Banks, 1983).

Acetaldehyde, an avid sulphite binding compound, was detected in lamb burgers at a concentration of 60 μg g^{-1} meat (Fig. 26c). This accounted for 34% of the sulphite bound even though 96% was bound after 6 d storage at 5°C (Fig. 26c).

Detection of Acetaldehyde Producing Yeasts

A medium was devised containing glucose and Schiff's reagent (2.5 g l^{-1} sodium sulphite and 0.4 g l^{-1} basic fuchsin) to detect acetaldehyde producing yeasts. Basic fuchsin bound to sodium sulphite resulted in a colourless medium. When 20 μl of solutions of avid sulphite binding compounds were inoculated onto the medium acetaldehyde and 2-oxoglutaric acid were found to be the most efficient at restoring the red colouration (Plate 3). The yeast colonies that restored the red colouration (Plate 4) to the medium

Plate 3. The reaction of chemicals inoculated onto acetaldehyde detection medium.

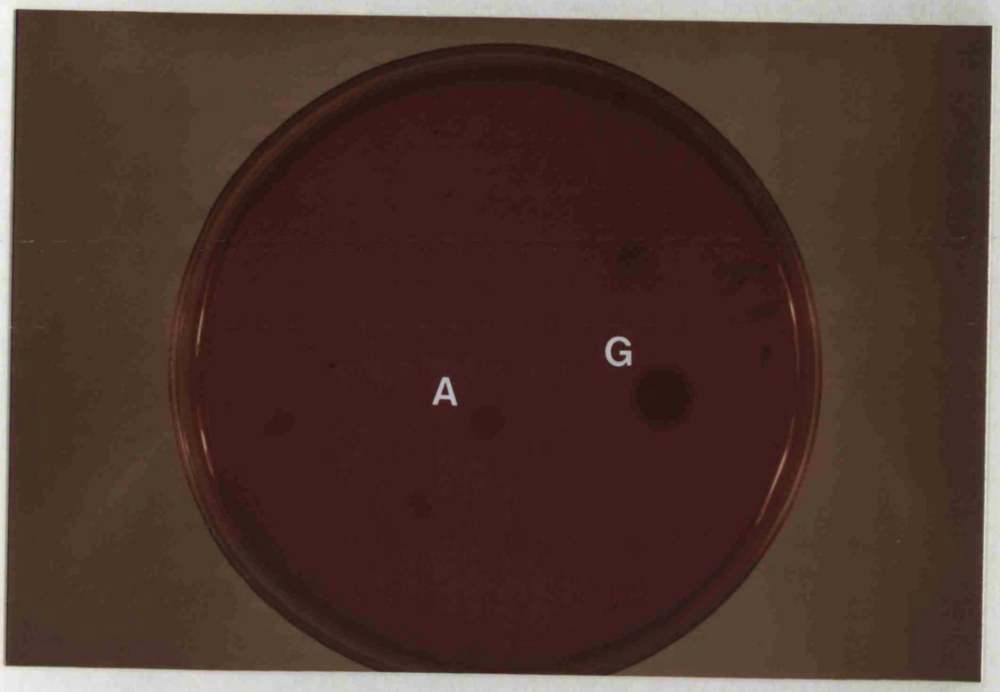
A Acetaldehyde

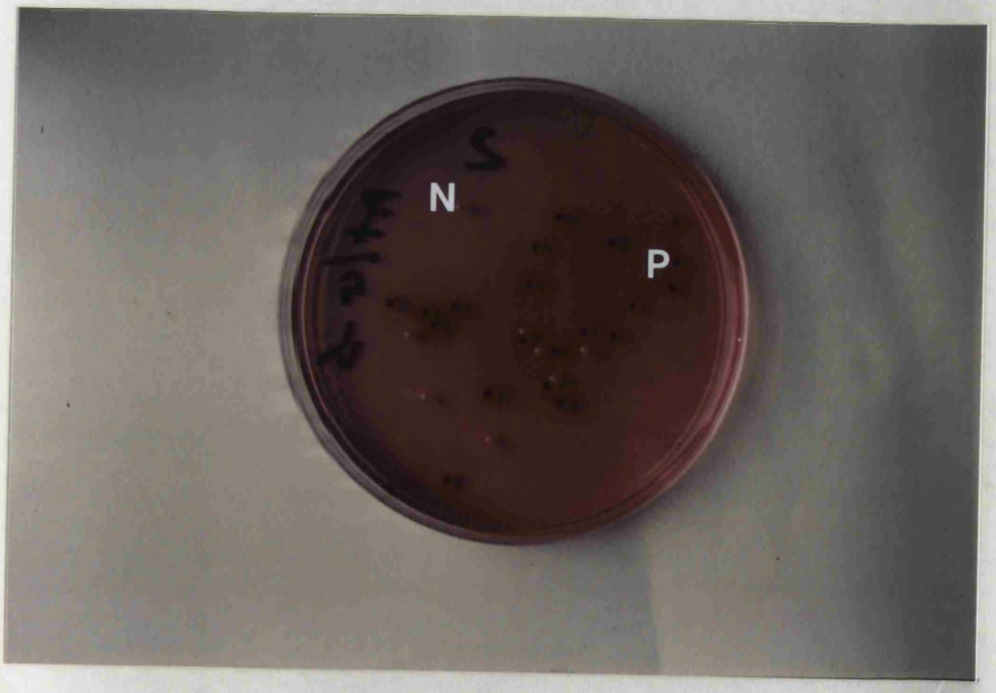
G 2-oxoglutaric acid

Plate 4. The reaction of yeasts on acetaldehyde detection medium incubated at 25°C for 5 d.

P positive (acetaldehyde producer)
sulphite binder

N negative
non-sulphite binder


A
G


N
P

were recorded as positive acetaldehyde producers (sulphite binders).

Schiff's reagent incorporated into OGYA was used to enumerate the number of acetaldehyde producing yeasts present in sulphited minced lamb stored at 5°C. Sixty percent of the initial yeast population were acetaldehyde producers (potential sulphite binders). The loss of free sulphite with storage was accompanied by an increase in the numbers of non-acetaldehyde producing yeasts (Fig. 19).

Thirty eight percent of the meat isolates multipoint inoculated onto the detection medium were found to be positive acetaldehyde producers. These were oxidative or weakly fermentative yeasts. Indeed 14% of the meat isolates fermented glucose (Table 14).

When yeasts that were positive acetaldehyde producers on glucose containing medium were inoculated onto lactate medium they failed to restore the red colouration to the agar. A feature supported by studies with broth cultures.

From the detection medium, a negative and a positive acetaldehyde producer (a non-binder and a sulphite binder) were selected. The non-binder was identified with Candida vini and the sulphite binder with Candida norvegica. These two yeasts, isolated from lamb burgers, were used to study sulphite binding and acetaldehyde production in pure broth cultures.

It was evident from the result discussed above that sulphite in lamb products selected Gram-positive bacteria (Br. thermosphacta and homofermentative lactobacilli) and yeasts, as is the case also in the British fresh sausage (Brown, 1977; Banks, 1983; Dalton,

1984). Additionally, when compared to the total viable counts, yeasts were found to be numerically dominant in preserved minced lamb. A percentage of these yeasts were noted to produce acetaldehyde, a compound responsible for the increased sulphite binding and hence the decreased antimicrobial activity of the preservative.

3. Sulphite Tolerance of Yeasts in Broth Cultures

The pH determines the proportions of molecular sulphur dioxide (SO_2), bisulphite (HSO_3^-) and sulphite (SO_3^{2-}) ions (Fig. 4) present in solution and hence the efficacy of sulphite as an antimicrobial agent (Hammond and Carr, 1976; King et al., 1981). As was evident in the literature review (Section 5), the resistance of yeasts to molecular sulphur dioxide in wine (pH 2.8 - 4.2) has been studied extensively whereas the tolerance of yeasts to bisulphite and sulphite ions present in meat (pH 5.8 - 6.8) has been largely ignored. Dalton (1984), however, noted that yeasts isolated from sulphited sausages produced acetaldehyde (as do wine yeasts) that was the main cause of sulphite binding in meat. This study was undertaken therefore to confirm whether or not yeasts from lamb products also produced acetaldehyde and to investigate the effect of pH, sulphite concentration and substrate availability on the sulphite tolerance of yeasts.

Two yeasts isolated from lamb burgers were selected from the acetaldehyde detection medium (results - Section 2). One, Candida norvegica, was an acetaldehyde producer. The other, Candida vini, was negative on the acetaldehyde detection medium. Lab lemco broth,

with glucose or lactate as substrates, was used as it simulated meat in terms of its chemical composition.

The proportions of molecular sulphur dioxide, bisulphite and sulphite ions vary with pH and it is therefore essential to maintain a stable pH when studying sulphite tolerance of microorganisms. Preliminary observations showed that unbuffered lab lemco glucose broth, inoculated with C. norvegica or C. vini, initially poised at pH 7 had a pH of 5.6 after incubation at 25°C for 24 h in a shaking water bath. Lab lemco glucose broth was therefore buffered with appropriate systems, inoculated with C. norvegica and incubated at 25°C for 20 h. The initial pH was not maintained during incubation with non-metabolisable buffers (MOPS and HEPES (pH 7), MES (pH 6) - Sigma). Succinic acid/sodium hydroxide and citric acid/sodium citrate buffer did not maintain the pH of the broth at pH 4 and 5 although the latter was more efficient at pH 6. Citrate phosphate buffer (sodium or potassium) maintained the pH of the broths at pH 4, 5, 6 and 7 over 24 h incubation at 25°C. The amount of sulphite bound, acetaldehyde produced and growth of meat yeasts was comparable with the sodium and potassium salts. Conversely with, a wine yeast, Zygosaccharomyces bailii, growth was reduced at pH 5 and completely inhibited at pH 6 and 7 with the sodium but not with the potassium buffered broth (Fig. 27a).

The growth of Z. bailii was retarded, however, in unsulphited or sulphited (100 µg SO_2 ml^{-1}) yeast extract glucose broth at pH 6 or 7 in comparison to the growth at pH 5 or 4 (Fig. 27a). The apparent reduced growth was associated with a lower percentage of

Figure 27. The effect of pH on growth, sulphite binding and acetaldehyde production by Zygosaccharomyces bailii (NCYC 1427) incubated at 25°C for 21 h.

a) Growth

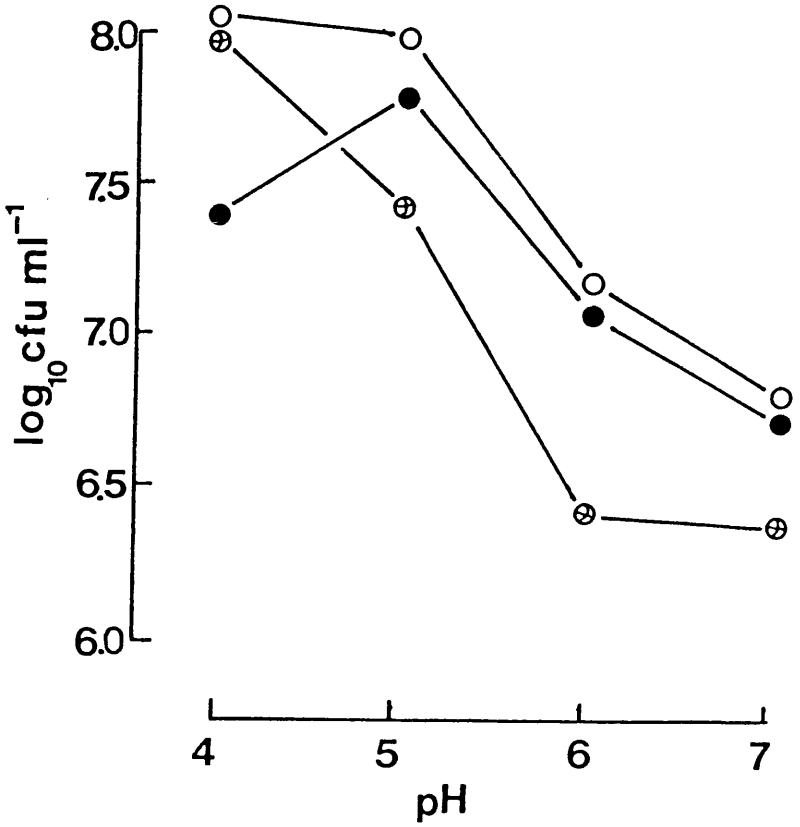
- ● Sulphited (potassium buffer)
- ○ Unsulphited (potassium buffer)
- ⊖ Unsulphited (sodium buffer)

b) Sulphite binding and acetaldehyde production

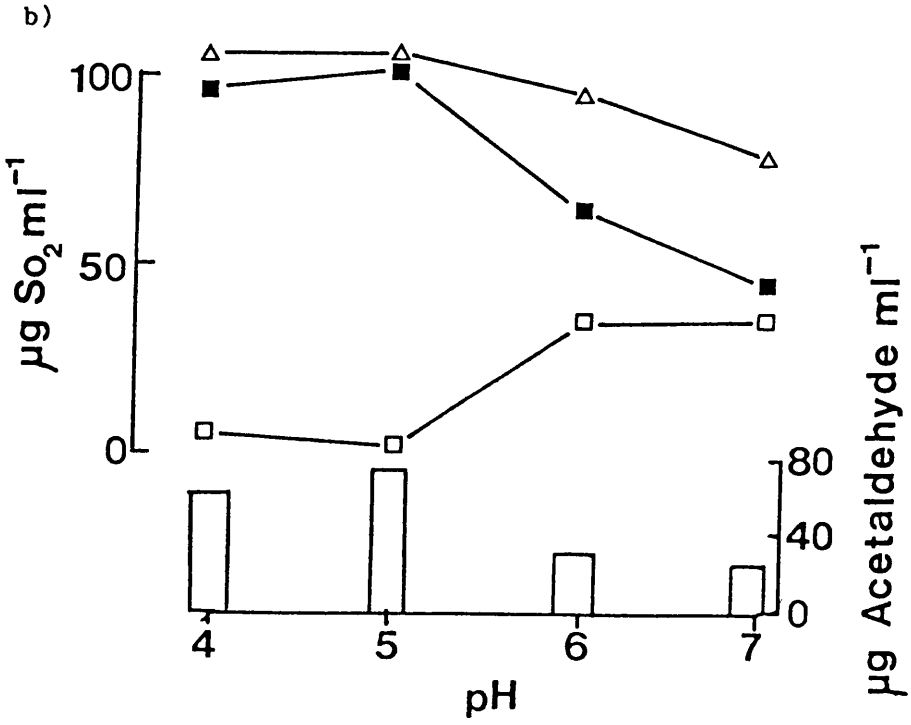
- Δ Total sulphite
- ■ Bound sulphite
- □ Free sulphite

Points - mean of two observations

a)


8.0
7.5
7.0
6.5
6.0
log10 cfu ml-1
4
5
6
7
pH

b)


100
50
0
µg So2 ml-1
80
40
0
µg Acetaldehyde ml-1
4
5
6
7
pH

bound sulphite (62%) as compared to 95% at pH 4 and 5 (Fig. 27b).

Growth

a) The Effect of pH

Ten yeasts were screened initially for sulphite tolerance (0 - 500 µg SO_2 ml^{-1}) over a pH range (3.5 - 7.0) in lab lemco glucose broth in microtitre plates. Of these, C. norvegica and C. vini were further tested 5 times in lab lemco glucose and lactate broth in microtitre plates and turbidity was recorded at time intervals over 72 h. The graphs (Figs. 28, 29) of turbidity readings at 48 h are representative of these results. There was no apparent inhibition of growth at pH 7 and 6 (500 µg SO_2 ml^{-1}) but inhibition occurred with increasing acidity and was accentuated by increasing sulphite concentration. Candida vini, however, was the most sensitive to these changes in pH and sulphite concentration.

The small volume (0.2 ml) in microtitre wells was not suitable for determining eventual sulphite concentration by the method of Banks and Board (1982a). To ensure accurate measurements of free and bound sulphite and acetaldehyde production, C. norvegica and C. vini were inoculated into 100 ml lab lemco broth in Erlenmeyer flasks, and incubated for at 25°C for 20 h in a shaking water bath. Turbidity in this instance was related to c.f.u.ml^{-1} by reference to a standard graph.

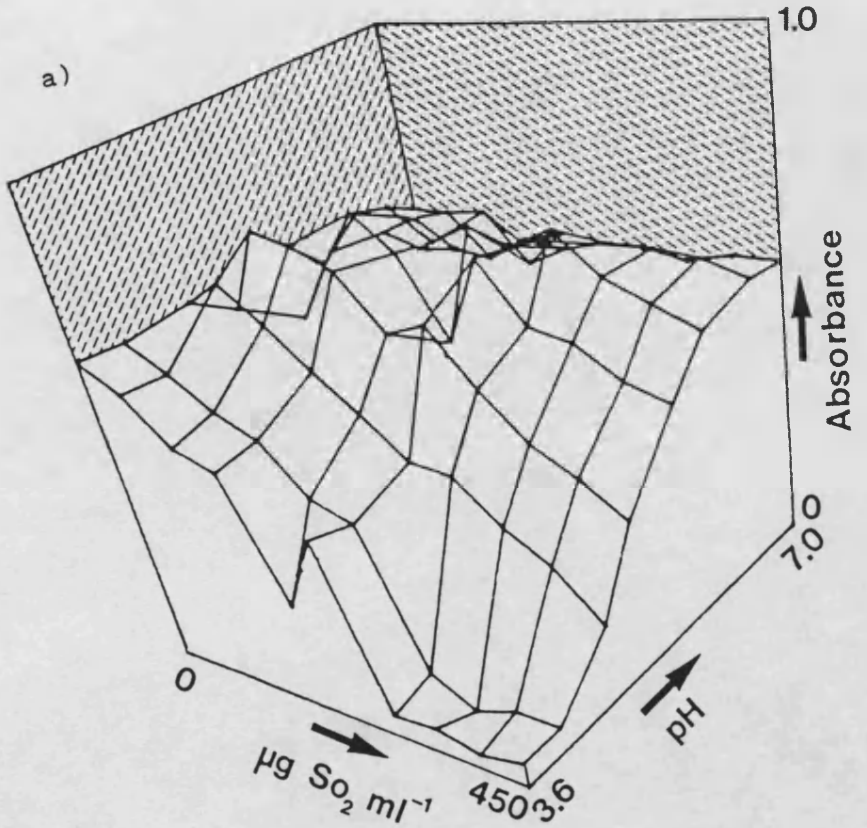
The effect of sulphite on the size of the yeast cell was investigated to determine whether or not it influenced turbidity measurements. The cell size of C. norvegica was significantly less in length and width at pH 4 and that of C. vini was significantly

Figure 28. The combined effect of pH and sulphite concentration on the growth of Candida norvegica in microtitre plates incubated at 25°C for 48 h:

a) in lab lemco glucose broth

b) in lab lemco lactate broth

3-D Graphics - M. Cole and M. Stratford.


a)
1.0
Absorbance
0
7.0
0
µg So_2 ml^{-1}
450
3.6
pH

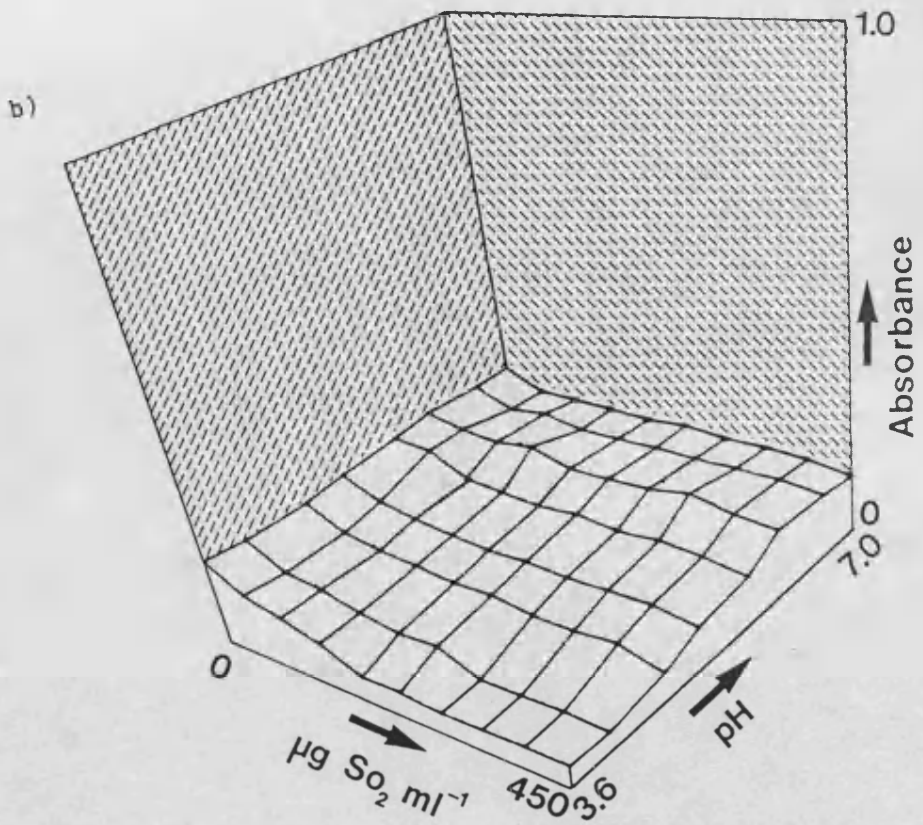

b)
1.0
Absorbance
0
7.0
0
µg So_2 ml^{-1}
450
3.6
pH

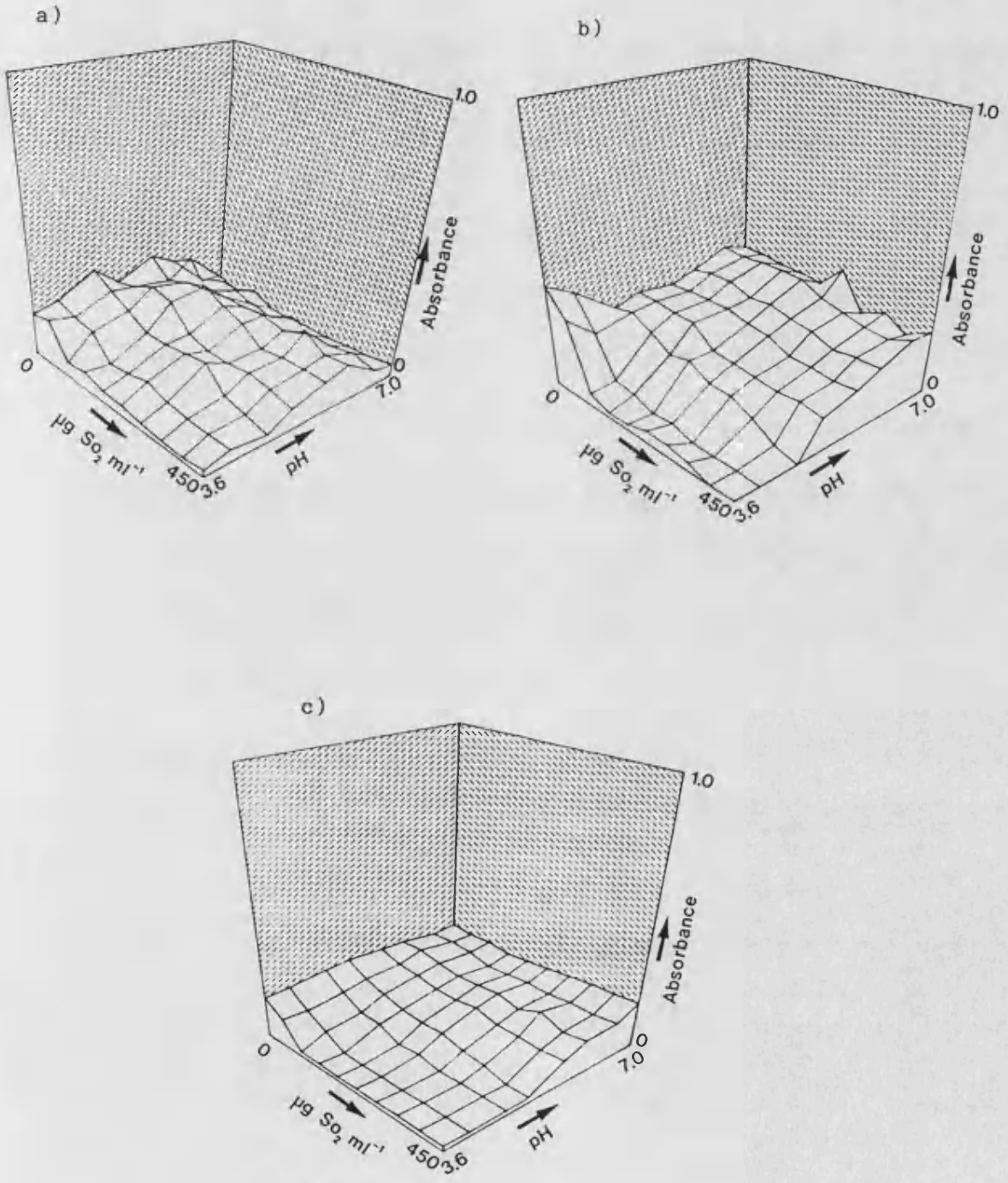
Figure 29. The combined effect of pH and sulphite concentration on the growth of yeasts in microtitre plates incubated at 25°C for 48 h:

a) Rhodotorula sp. in lab lemco glucose broth

b) Candida vini in lab lemco glucose broth

c) Candida vini in lab lemco lactate broth

3-D Graphics - M. Cole and M. Stratford.


a)
1.0
Absorbance
0
0
7.0
μg SO_2 ml^{-1}
450
3.6
pH
b)
1.0
Absorbance
0
0
7.0
μg SO_2 ml^{-1}
450
3.6
pH
c)
1.0
Absorbance
0
0
7.0
μg SO_2 ml^{-1}
450
3.6
pH

shorter at pH 5, in sulphited lab lemco glucose broth when compared with unsulphited cultures (Table 22). At these pH values turbidity did not increase (as compared to initial density). This was supported by the fact that only negligible amounts of dry cell weights were recovered (Fig. 30). The cells of C. vini were significantly wider in sulphited (pH 6) broth as compared to unsulphited cultures (Table 22). Similar dry cell weights were recovered with C. vini and C. norvegica at pH 6 (Fig. 30) and there was no significant difference (Tables 24, 25, 29) in growth (related to turbidity). These results indicate that the small difference in cell size induced by sulphite did not affect the accuracy of growth measurements.

b) The Effect of Sulphite Concentration

Sulphite concentrations up to 1000 µg SO_2 ml^{-1} did not affect the growth of C. norvegica or C. vini in lab lemco glucose broth cultures buffered at pH 7 (Fig. 31d). Inhibition increased with decreasing pH and was accentuated by increasing sulphite concentration. Again, C. vini was noted to be most sensitive to these changes in pH and sulphite concentration. Thus, at pH 6 the eventual population size of C. norvegica and C. vini was reduced with 750 µg SO_2 ml^{-1} and that of the latter was further reduced at 1000 µg SO_2 ml^{-1} when compared to unsulphited cultures (Fig. 31c).

At pH 5 the reduction in the eventual population size of C. norvegica was directly related to the increase in sulphite concentration (Fig. 31b). Growth of this organism, however, was completely inhibited by 250 µg SO_2 ml^{-1} at pH 4 (Fig. 31a). In

Table 22. Cell size measurements at pH 4, 5, 6 and 7 in sulphited or unsulphited lab lemco glucose broth.

		pH 4	pH 5	pH 6	pH 7
Candida norvegica					
Unsulphited		4.6* x 2.90 ± 0.1 ± 0.1	4.67 x 2.84 ± 0.17 ± 0.08	4.53 x 2.73 ± 0.13 ± 0.06	4.40 x 2.72 ± 0.13 ± 0.07
Sulphited		3.72 x 2.36 ± 0.13 ± 0.05	4.66 x 2.75 ± 0.16 ± 0.05	4.64 x 2.57 ± 0.12 ± 0.07	4.8 x 2.77 ± 0.17 ± 0.07
t test	length	4.56** P<0.001	0.04 NSD	0.6 NSD	1.9 NSD
	width	5.8 P<0.001	0.9 NSD	1.6 NSD	0.5 NSD
Candida vini					
Unsulphited		4.94 x 3.19 ± 0.17 ± 0.06	5.25 x 3.46 ± 0.20 ± 0.07	5.15 x 3.33 ± 0.18 ± 0.07	5.1 x 3.28 ± 0.20 ± 0.08
Sulphited		4.54 x 3.32 ± 0.19 ± 0.09	4.21 x 3.3 ± 0.15 ± 0.07	5.37 x 3.71 ± 0.19 ± 0.07	5.12 x 3.40 ± 0.17 ± 0.08
t test	length	1.57 NSD	4.09 P<0.001	0.83 NSD	0.07 NSD
	width	1.09 NSD	1.46 NSD	3.6 P<0.001	1.05 NSD

* Cell size - mean length x width (µm) ± standard error n = 50

** t values

Figure 30. Dry cell weights recovered from lab lemco glucose broth cultures incubated at 25°C for 20 h.

N *Candida norvegica*

V *Candida vini*

Unsulphited

Sulphited (500 µg SO_2 ml^{-1})

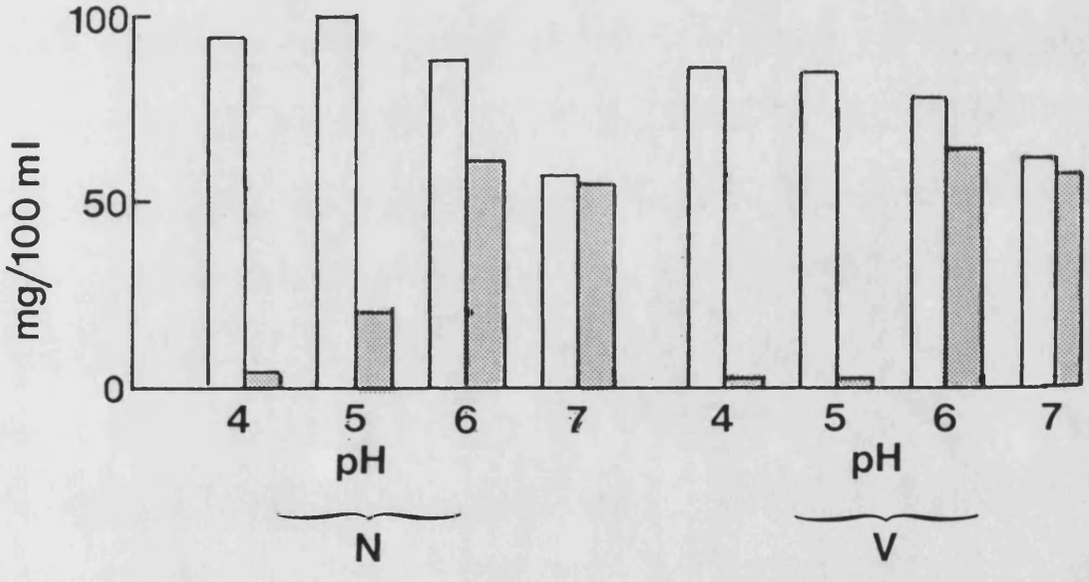

100
50
0
mg/100 ml
4
5
6
7
pH
N
4
5
6
7
pH
V

Figure 31. The effect of sulphite concentration on the growth of yeasts in lab lemco glucose broth incubated at 25°C for 20 h :

a) pH 4

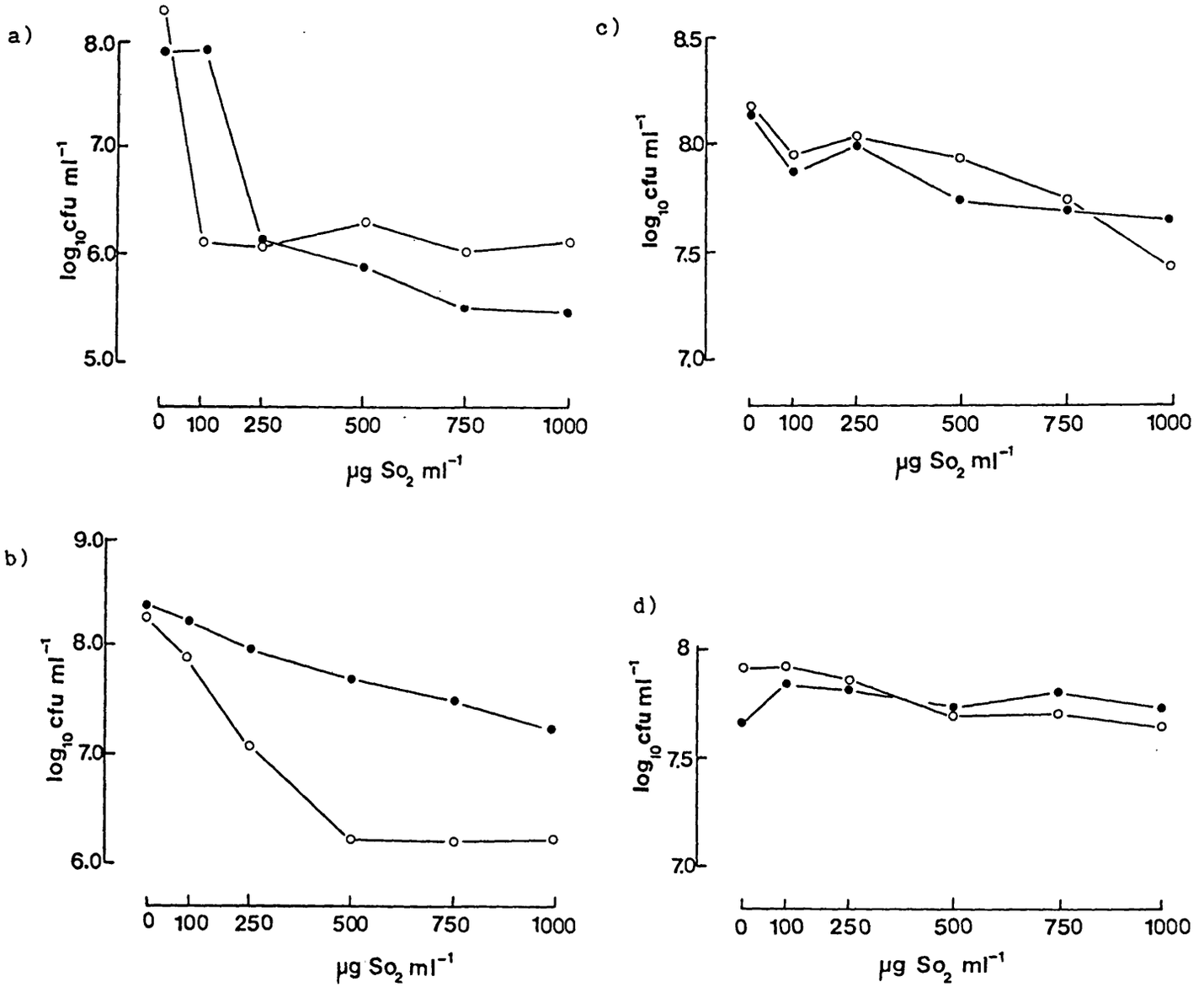
b) pH 5

c) pH 6

d) pH 7

● Candida norvegica

○ Candida vini


a)
8.0
7.0
6.0
5.0
log10cfu ml-1
0 100 250 500 750 1000
µg So2 ml-1
c)
8.5
8.0
7.5
7.0
log10cfu ml-1
0 100 250 500 750 1000
µg So2 ml-1
b)
9.0
8.0
7.0
6.0
log10cfu ml-1
0 100 250 500 750 1000
µg So2 ml-1
d)
8
7.5
7.0
log10cfu ml-1
0 100 250 500 750 1000
µg So2 ml-1

contrast, growth of C. vini was completely inhibited by 500 μg SO_2 ml^{-1} at pH 5 and 100 μg SO_2 ml^{-1} at pH 4 (Fig. 31a, b).

c) The Effect of Substrate

A survey of substrates, many of which could occur in meat, was set up to investigate the influence of substrates per se on sulphite tolerance of C. norvegica (Fig. 32). Sulphite depressed the amount of growth at 20 h of C. norvegica in lab lemco broth buffered at pH 6 and supplemented with (0.5 - 2% w/v): cellobiose, glycerol, 3-O-methyl glucose, lactate, maltose, salicin, sorbitol, sorbose, sucrose or starch. Notably less inhibition of growth was associated with a greater loss of free suphite. This loss of preservative occurred by: 1) irretrievable loss presumably by oxidation to sulphate (greatly enhanced with acetate or succinate); 2) loss by binding a) immediately to the substrate (as with pyruvate), or b) with growth of C. norvegica (as with ethanol, fructose or glucose). The concentration of glucose (0.1 - 2% w/v) was unimportant as regards sulphite tolerance of C. norvegica, but glucose was noted to maintain the total sulphite concentration (Figs. 33, 46).

Of these compounds, glucose is the most readily available substrate in meat. This was therefore used to investigate sulphite tolerance of C. norvegica and C. vini over the pH range 4-7. Inoculum was from lab lemco glucose broth cultures.

Sulphite (500 μg SO_2 ml^{-1}) had no effect on the rate (doubling time) or extent of growth of C. norvegica or C. vini in lab lemco glucose broth buffered at pH 7 (Table 23, Fig. 35). Thus, there was

Figure 32. The effect of substrates at pH 6 on the growth of Candida norvegica in lab lemco broth incubated at 25°C for 20 h.

1	Acetate	9	Sorbose
2	Cellobiose	10	Succinate
3	Glycerol	11	Sucrose
4	3-O-methyl glucose	12	Starch
5	Lactate	13	Pyruvate
6	Maltose	14	Fructose
7	Salicin	15	Glucose
8	Sorbitol	16	Ethanol

□ Unsulphited ▒ Sulphited (500 µg SO_2 ml^{-1})

Mean of 2-5 observations

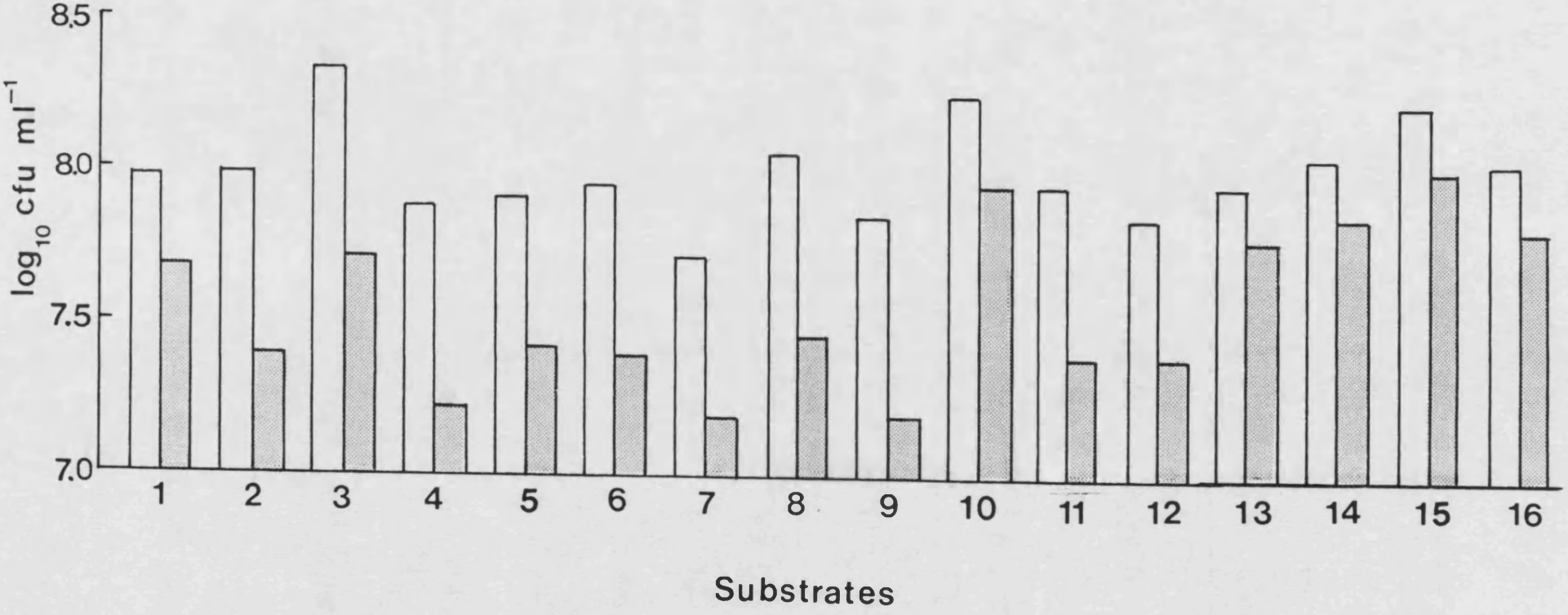

8.5
8.0
7.5
7.0
log10 cfu ml-1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Substrates

Figure 33. The effect of glucose concentration at pH 6 on the growth of Candida norvegica in lab lemco broth incubated at 25°C for 20 h.

○ Unsulphited

● Sulphited (500 μg SO_2 ml^{-1})

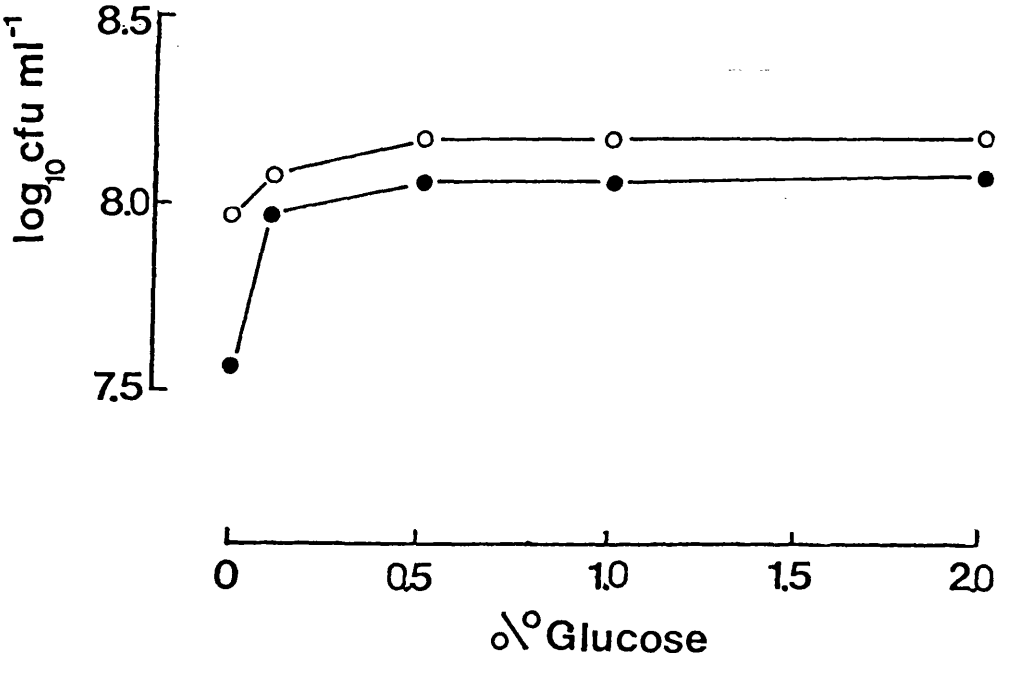

8.5
8.0
7.5
log10 cfu ml-1
0
0.5
1.0
1.5
2.0
% Glucose

Figure 34. The growth of yeasts in lab lemco glucose broth incubated at 25°C:

Candida norvegica	a)	pH 4
	b)	pH 5
	c)	pH 6
Candida vini	d)	pH 4
	e)	pH 5
	f)	pH 6

○ Unsulphited

● Sulphited (500 μg SO_2 ml^{-1})

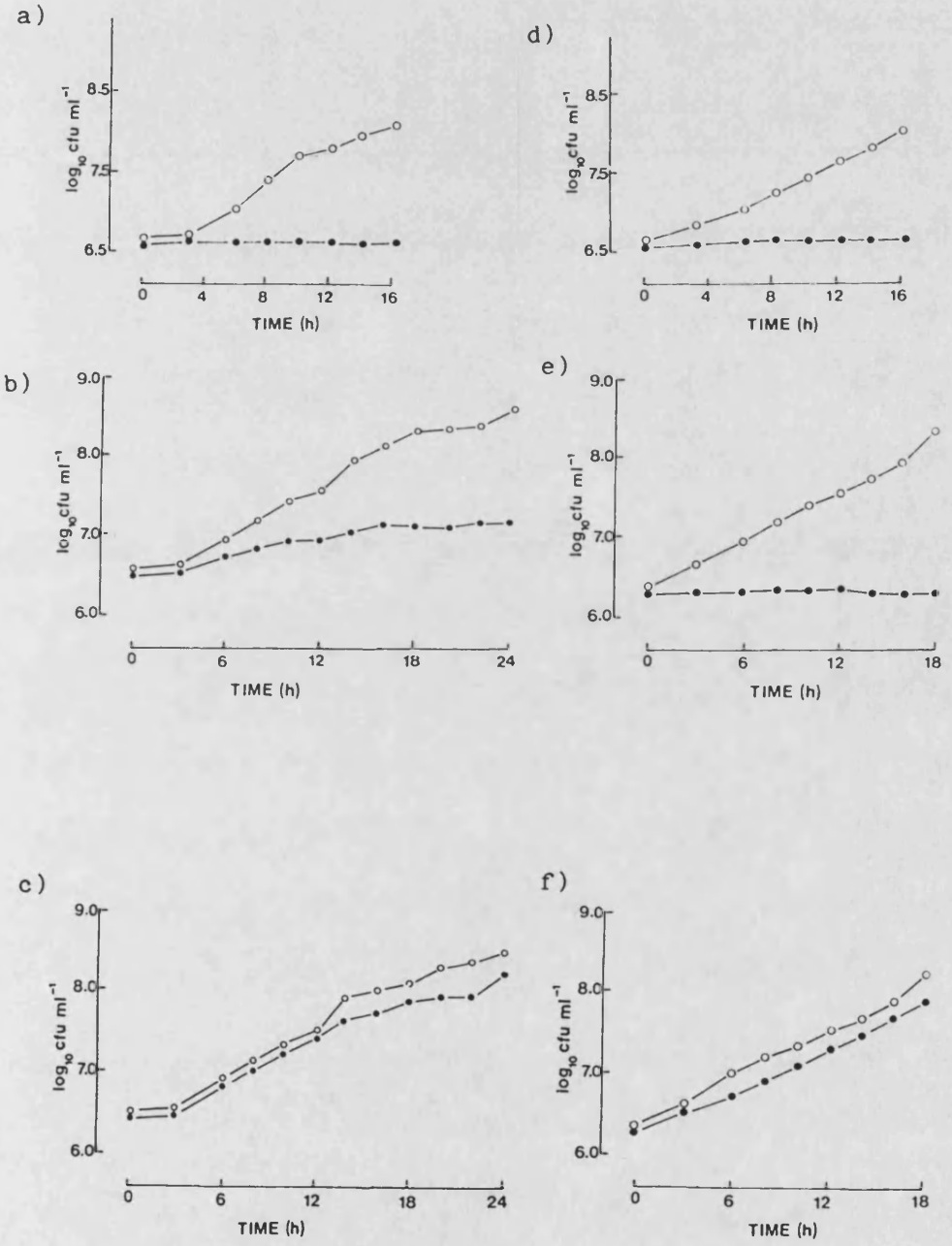

a)
log10 cfu ml-1
8.5
7.5
6.5
0
4
8
12
16
TIME (h)
d)
log10 cfu ml-1
8.5
7.5
6.5
0
4
8
12
16
TIME (h)
b)
log10 cfu ml-1
9.0
8.0
7.0
6.0
0
6
12
18
24
TIME (h)
e)
log10 cfu ml-1
9.0
8.0
7.0
6.0
0
6
12
18
TIME (h)
c)
log10 cfu ml-1
9.0
8.0
7.0
6.0
0
6
12
18
24
TIME (h)
f)
log10 cfu ml-1
9.0
8.0
7.0
6.0
0
6
12
18
TIME (h)

Figure 35. The growth of yeasts in lab lemco glucose broth incubated at 25°C:

Unbuffered broth initially poised at pH 7 reduced to pH 5.6 after 24 h.

a) Candida norvegica

b) Candida vini

Broth buffered at pH 7

c) Candida norvegica

d) Candida vini

○ Unsulphited

● Sulphited (500 μg SO_2 ml^{-1})

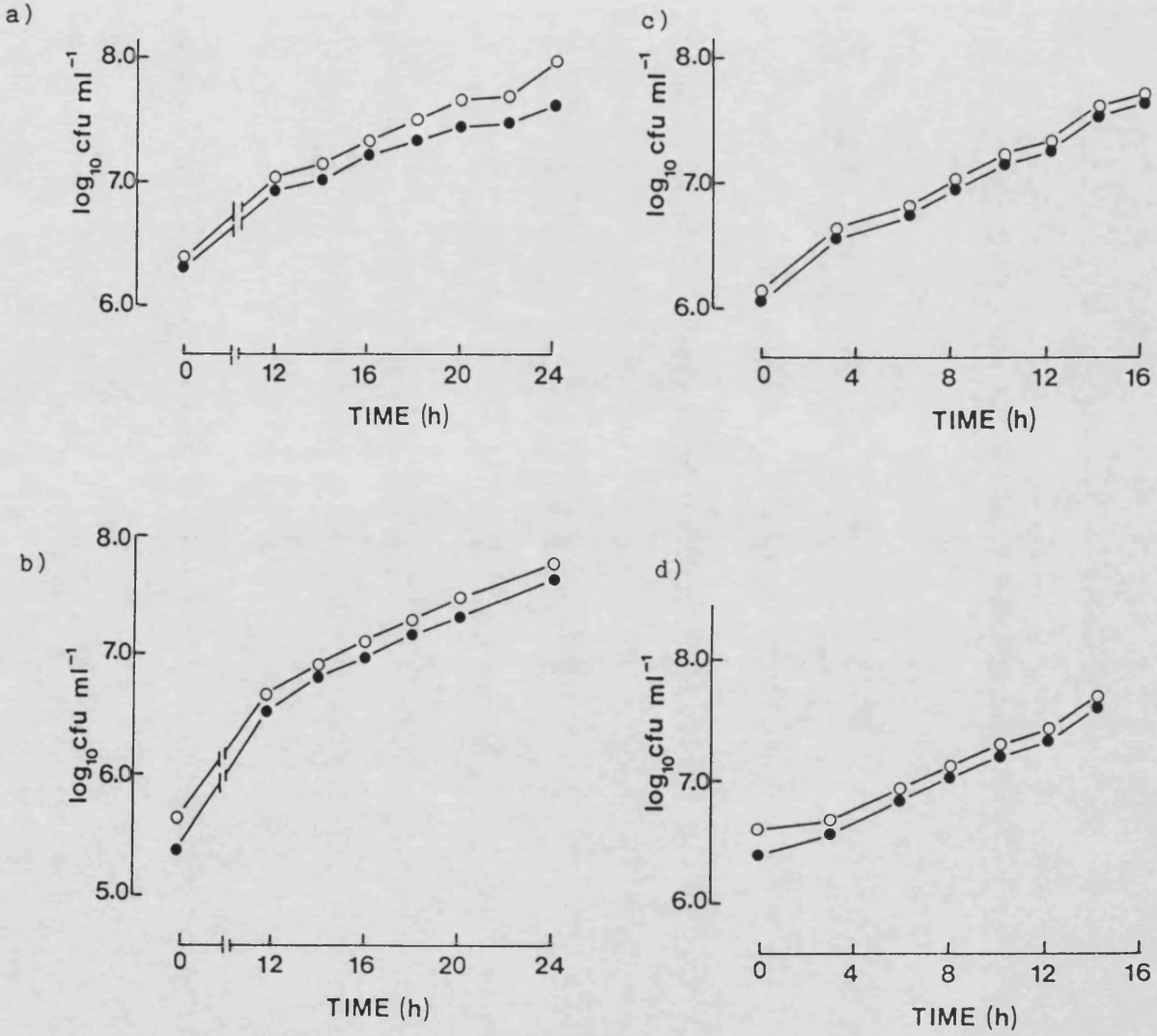

a)
8.0
7.0
6.0
log10 cfu ml-1
0
12
16
20
24
TIME (h)
c)
8.0
7.0
6.0
log10 cfu ml-1
0
4
8
12
16
TIME (h)
b)
8.0
7.0
6.0
5.0
log10cfu ml-1
0
12
16
20
24
TIME (h)
d)
8.0
7.0
6.0
log10cfu ml-1
0
4
8
12
16
TIME (h)

Table 23. Doubling times (h) of Candida norvegica and Candida vini in lab lemco glucose broth buffered with sodium citrate phosphate buffer.

	Candida norvegica		Candida vini	
pH	Unsulphited	Sulphited	Unsulphited	Sulphited
4	2.89	NG	3.32	NG
5	2.80	6.43	2.81	NG
6	3.05	3.66	2.90	3.26
7	3.11	3.16	3.42	3.49
Unbuffered	3.69	4.22	3.21	3.18

NG - no detectable growth after 72 h.

no significant difference in growth of these yeasts in sulphited compared to unsulphited broths (Table 29) or C. norvegica vis à vis C. vini (Table 30a).

At pH 6 there was no difference in growth in unsulphited vis à vis unsulphited broth, during the lag phase with C. norvegica but less growth occurred throughout the exponential phase, a trend that was more notable at pH 5. This trend was accentuated with C. vini. The generation time of C. norvegica and C. vini was extended in sulphited lab lemco glucose broth buffered at pH 6, compared to unsulphited broth (Table 23) but the reduction in the final population sizes, however, was not significant (Tables 24, 25, 29, Figs 34c, f). Thus, the growth of C. norvegica and C. vini in sulphited broth at pH 6 was equal to that at pH 7 (Table 26).

The generation time of C. norvegica was doubled in the presence of sulphite in lab lemco glucose broth buffered at pH 5 (Table 23) and this reduced significantly the final population size (Table 29, Fig. 34b). In contrast, the growth of C. vini was completely inhibited (i.e. no increase above the initial inoculum level) in sulphited broth at pH 5 (Fig. 34e, Table 29) and hence was significantly less than that in the unsulphited broth (Table 29), and that of C. norvegica (Table 30a). Thus, sulphite significantly reduced the growth of C. norvegica and C. vini at pH 5 when compared to that at pH 7 and 6 (Table 26).

The effect of sulphite on growth was accentuated at pH 4. Candida norvegica and C. vini were completely inhibited in sulphited lab lemco glucose broth buffered at pH 4 (Figs. 34a,d, Tables 24, 25) i.e. no growth was evident after 72 h.

Table 24. Amount of sulphite bound, acetaldehyde produced and growth of Candida norvegica at pH 4, 5, 6 and 7 with different substrates (means and standard errors of 5 experiments). Incubation for 20 h at 25°C. *

	Growth (unsulphited) x 10^7 cfu ml^{-1}	Growth (sulphited) x10^7 cfu ml^{-1}	Sulphite bound µg ml^{-1}	Acetaldehyde µg ml^{-1}
Glucose				
pH 4	14.85 ± 2.10	0.37 ± 0.05	97.7 ± 10.7	0.5 ± 0.4
5	16.76 ± 1.59	2.30 ± 0.31	212.0 ± 33.5	77.60 ± 23.5
6	13.61 ± 2.04	8.45 ± 1.25	358.0 ± 31.1	207.1 ± 7.4
7	11.14 ± 3.02	9.80 ± 2.06	123.0 ± 31.7	40.4 ± 18.9
Fructose				
pH 4	12.70 ± 0.60	0.08 ± 0.02	61.0 ± 4.56	0.1 ± 0.1
5	12.19 ± 2.10	1.77 ± 0.40	134.6 ± 38.1	45.0 ± 21.3
6	9.57 ± 0.45	7.32 ± 1.30	319.0 ± 34.1	175.2 ± 9.4
7	7.12 ± 0.54	6.19 ± 0.92	132.0 ± 18.8	45.3 ± 10.1
Ethanol				
pH 4	7.47 ± 0.36	0.09 ± 0.02	22.0 ± 2.1	0 ± 0
5	8.53 ± 0.63	2.68 ± 0.35	526.6 ± 16.1	504.8 ± 42.9
6	9.58 ± 0.76	6.00 ± 0.49	425.6 ± 24.4	352.0 ± 53.4
7	5.83 ± 0.66	5.75 ± 0.45	381.0 ± 37.9	407.0 ± 90.2
Lactate				
pH 4	7.57 ± 2.25	0.21 ± 0.03	24.3 ± 3.5	1.1 ± 1.0
5	11.02 ± 1.18	1.44 ± 0.18	58.6 ± 23.4	2.5 ± 0.6
6	9.08 ± 0.95	3.90 ± 0.85	25.2 ± 3.3	2.8 ± 0.3
7	7.54 ± 0.70	8.90 ± 0.34	25.5 ± 2.5	0.2 ± 0.2

* Statistical analysis of these figures are given in Tables 26-30.

Table 25. Amount of sulphite bound, acetaldehyde produced and growth of Candida vini at pH 4, 5, 6 and 7 with different substrates (means and standard errors of 5 experiments). Incubation for 20 h at 25°C. *

	Growth (unsulphited) x 10^7 cfu ml^{-1}	Growth (sulphited) x10^7 cfu ml^{-1}	Sulphite bound µg ml^{-1}	Acetaldehyde µg ml^{-1}
Glucose				
pH 4	15.23 ± 5.02	0.41 ± 0.02	45.9 ± 22.5	0.1 ± 0.1
5	13.57 ± 1.55	0.22 ± 0.12	95.7 ± 6.3	0.3 ± 0.3
6	9.88 ± 1.23	3.93 ± 1.24	98.0 ± 29.1	4.5 ± 2.5
7	14.01 ± 4.25	11.60 ± 4.75	31.7 ± 16.8	5.1 ± 1.3
Lactate				
pH 4	5.75 ± 1.46	0.12 ± 0.05	19.6 ± 2.9	0.3 ± 0.2
5	8.75 ± 0.18	0.08 ± 0.02	24.7 ± 0.7	0.1 ± 0.1
6	8.86 ± 0.47	1.87 ± 0.31	27.7 ± 3.9	1.8 ± 1.0
7	7.01 ± 0.72	6.60 ± 0.26	29.0 ± 0.0	0.2 ± 0.2

* Statistical analysis of these figures are given in Tables 26, 28, 29, 30.

Table 26. Analysis of variance and significance tests[1] between sulphite bound, acetaldehyde production and growth at pH 4, 5, 6 and 7 for each substrate.

A) Candida norvegica

Substrate	Growth	Sulphite Bound	Acetaldehyde
Glucose	10.3[§] ***	22.6 ***	29.2 ***
	†4 < 5 < 6 = 7	4 = 7 < 5 < 6	4 < 7 = 5 < 6
Fructose	10.6 ***	11.4 ***	11.2 ***
	4 < 5 < 7 = 6	4 < 7 = 5 < 6	4 < 5 = 7 < 6
Ethanol	47.4 ***	77.9 ***	13.9 ***
	4 < 5 < 7 = 6	4 < 7 < 6 < 5	4 < 6 = 7 = 5
Lactate	21.0 ***	2.0 NSD	3.0 NSD
	4 < 5 < 6 = 7	4 = 6 = 7 = 5	7 = 4 = 5 = 6

1 Statistical analysis (Sokal and Rohlf, 1969)

§ Statistic F (Analysis of Variance)

† Multiple Range Test values given in ascending order

* P< 0.05; ** P< 0.01; *** P < 0.001

NSD Not significantly different, p > 0.05

Table 26. continued

B) Candida vini

Substrate	Growth	Sulphite Bound	Acetaldehyde
Glucose	6.1 § *	3.1 NSD	1.9 NSD
	† 5 = 4 < 6 = 7	7 = 4 = 5 = 6	4 = 5 = 6 = 7
Lactate	168 ***	2.2 NSD	1.7 NSD
	5 = 4 < 6 < 7	4 = 5 = 6 = 7	5 = 7 = 4 = 6

1 Statistical analysis (Sokal and Rohlf, 1969)

§ Statistic F (Analysis of Variance)

† Multiple Range Test values given in ascending order

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

NSD Not significantly different, $p > 0.05$

Table 27. Analysis of variance and significance tests between sulphite bound, acetaldehyde production and growth of Candida norvegica with glucose, fructose, ethanol and lactate at different pH.

pH	Growth	Sulphite Bound	Acetaldehyde
4	0.1[§] NSD †F = E = L = G	17.5 *** E = L < F < G	0.67 NSD E = F = G = L
5	3.18 NSD L = F = G = E	6.3 *** L = F F = G < E	79.8 *** L = F F = G < E
6	2.53 NSD L = E = F = G	48.7 *** L < F = G < E	2.11 *** L < F = G < E
7	1.3 NSD E = F = L = G	18.6 *** L < G = F < E	17.5 *** L = G = F < E

[§] Statistic F (Analysis of Variance)

† Multiple Range Test values given in ascending order

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

NSD No significant difference, $p > 0.05$

L = Lactate; F = Fructose; G = Glucose; E = Ethanol

N.B. Growth of C. vini with glucose vs lactate was not significantly different ($P > 0.05$)

Table 28. Comparison (t test) of bound sulphite in inoculated and uninoculated broths.

pH	Substrate	*Candida norvegica*		*Candida vini*	
		t value	significance	t value	significance
4	Lactate	0.5	NSD	1.5	NSD
	Glucose	1.5	NSD	0.62	NSD
	Fructose	0.6	NSD		
	Ethanol	1.2	NSD		
5	Lactate	0.62	NSD	0.8	NSD
	Glucose	3.5	*	1.52	NSD
	Fructose	2.1	NSD		
	Ethanol	18.2	***		
6	Lactate	0.24	NSD	0.7	NSD
	Glucose	9.7	***	1.57	NSD
	Fructose	8.5	***		
	Ethanol	16.1	***		
7	Lactate	0.69	NSD	3.7	NSD
	Glucose	2.37	*	0.25	NSD
	Fructose	5.5	**		
	Ethanol	9.5	***		

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$
NSD No significant difference $P > 0.05$

Table 29. Comparison (t test) of growth in sulphited and unsulphited broth.

pH	Substrate	*Candida norvegica*		*Candida vini*	
		t value	significance	t value	significance
4	Lactate	3.3	*	3.81	*
	Glucose	5.5	**	2.9	*
	Fructose	20.5	***		
	Ethanol	19.9	***		
5	Lactate	10.3	***	46.7	***
	Glucose	8.9	***	15.3	***
	Fructose	4.8	**		
	Ethanol	8.46	***		
6	Lactate	4.1	**	12.4	***
	Glucose	2.1	NSD	1.4	NSD
	Fructose	1.4	NSD		
	Ethanol	3.95	**		
7	Lactate	1.7	NSD	1.48	NSD
	Glucose	0.37	NSD	0.4	NSD
	Fructose	0.9	NSD		
	Ethanol	0.1	NSD		

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$
NSD No significant difference $P > 0.05$

Table 30a. Comparison (t test) of sulphite bound, acetaldehyde production and growth between Candida norvegica and Candida vini with glucose as substrate.

pH		Growth	Sulphite Bound	Acetaldehyde
4	C. norvegica v C. vini	1.2§	2.2	0.68
		NSD	NSD	NSD
5	C. norvegica v C. vini	4.9	2.6	2.8
		**	*	*
6	C. norvegia v C. vini	1.8	5.9	22.4
		NSD	**	***
7	C. norvegica v C. vini	0.4	2.08	1.2
		NSD	NSD	NSD

§ t values

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

NSD No significant difference $P > 0.05$

Table 30b. Comparison (t test) of sulphite bound, acetaldehyde produced and growth between Candida norvegica and Candida vini with lactate as substrate.

pH		Growth	Sulphite Bound	Acetaldehyde
4	C. norvegica v C. vini	1.5[§]	0.8	0.7
		NSD	NSD	NSD
5	C. norvegica v C. vini	5.7	1.4	2.3
		**	NSD	NSD
6	C. norvegica v C. vini	1.8	0.47	1.1
		NSD	NSD	NSD
7	C. norvegica v C. vini	3.7	1.4	0.2
		NSD	NSD	NSD

§ t values

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

NSD No significant difference $P > 0.05$

The sulphite tolerance of C. norvegica and C. vini over the pH range 4-7 was further tested with lactate, another substrate readily available in meat. There was no significant difference in growth of C. norvegica or C. vini in sulphited lab lemco lactate broth vis à vis sulphited glucose broth at pH 4, 5, 6 or 7 (Table 27, Figs. 36, 37a,b,d,e). Growth of these yeasts was unaffected by sulphite in lactate broth at pH 7 (Figs. 37d,e, Table 29) but was, however, significantly less at pH 6, 5 and 4 (Figs. 36, 37d,e, Table 29). Sulphite inhibition at pH 5 was thus accentuated with lactate as compared to glucose. The growth of C. norvegica at pH 5, however, was diminished by sulphite to the same extent with either substrates (Figs. 37a,b,d,e). Candida norvegica and C. vini were completely inhibited by sulphite at pH 4 and the latter at pH 5 (Figs. 37a,b,d,e) in lactate broth as was the case in glucose broth.

It was noted in the survey of substrates at pH 6, that sulphite binding occurred in lab lemco fructose and ethanol broth cultures of C. norvegica. These two substrates were therefore used to investigate the sulphite tolerance of C. norvegica over the pH range 4-7. The growth of C. norvegica in sulphited (500 µg SO_2 ml^{-1}) lab lemco fructose or ethanol broth at pH 4, 5, 6 or 7 was not significantly different from that obtained with glucose or lactate broth cultures (Table 27). Growth was unaffected by sulphite at pH 7 with either substrate or at pH 6 with fructose (Table 29). Sulphite inhibition at pH 6 was thus accentuated with ethanol as compared to fructose. Growth of C. norvegica at pH 5 was diminished by sulphite to a similar extent in fructose or ethanol

Figure 36. The growth of yeasts in lab lemco lactate broth incubated at 25°C:

Candida norvegica	a)	pH 4
	b)	pH 5
	c)	pH 6
Candida vini	d)	pH 4
	e)	pH 5
	f)	pH 6

○ Unsulphited

● Sulphited (500 µg SO_2 ml^{-1})

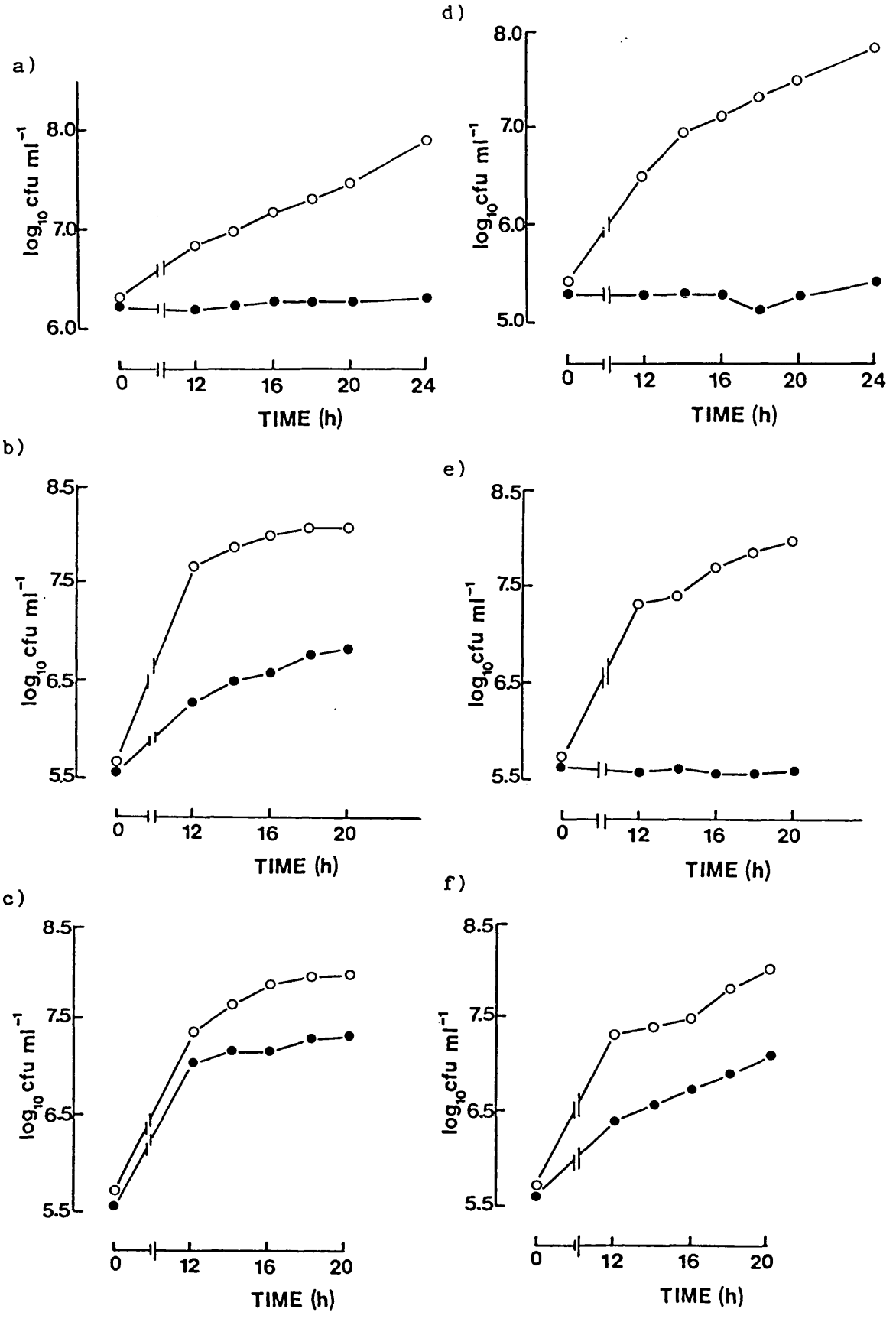

a)
8.0
7.0
6.0
log10 cfu ml-1
0
12
16
20
24
TIME (h)
d)
8.0
7.0
6.0
5.0
log10 cfu ml-1
0
12
16
20
24
TIME (h)
b)
8.5
7.5
6.5
5.5
log10 cfu ml-1
0
12
16
20
TIME (h)
e)
8.5
7.5
6.5
5.5
log10 cfu ml-1
0
12
16
20
TIME (h)
c)
8.5
7.5
6.5
5.5
log10 cfu ml-1
0
12
16
20
TIME (h)
f)
8.5
7.5
6.5
5.5
log10 cfu ml-1
0
12
16
20
TIME (h)

Figure 37. The effect of pH on the growth of yeasts in lab lemco broth incubated at 25°C for 20 h.

a) Glucose - Candida norvegica

b) Glucose - Candida vini

c) Fructose - Candida norvegica

d) Lactate - Candida norvegica

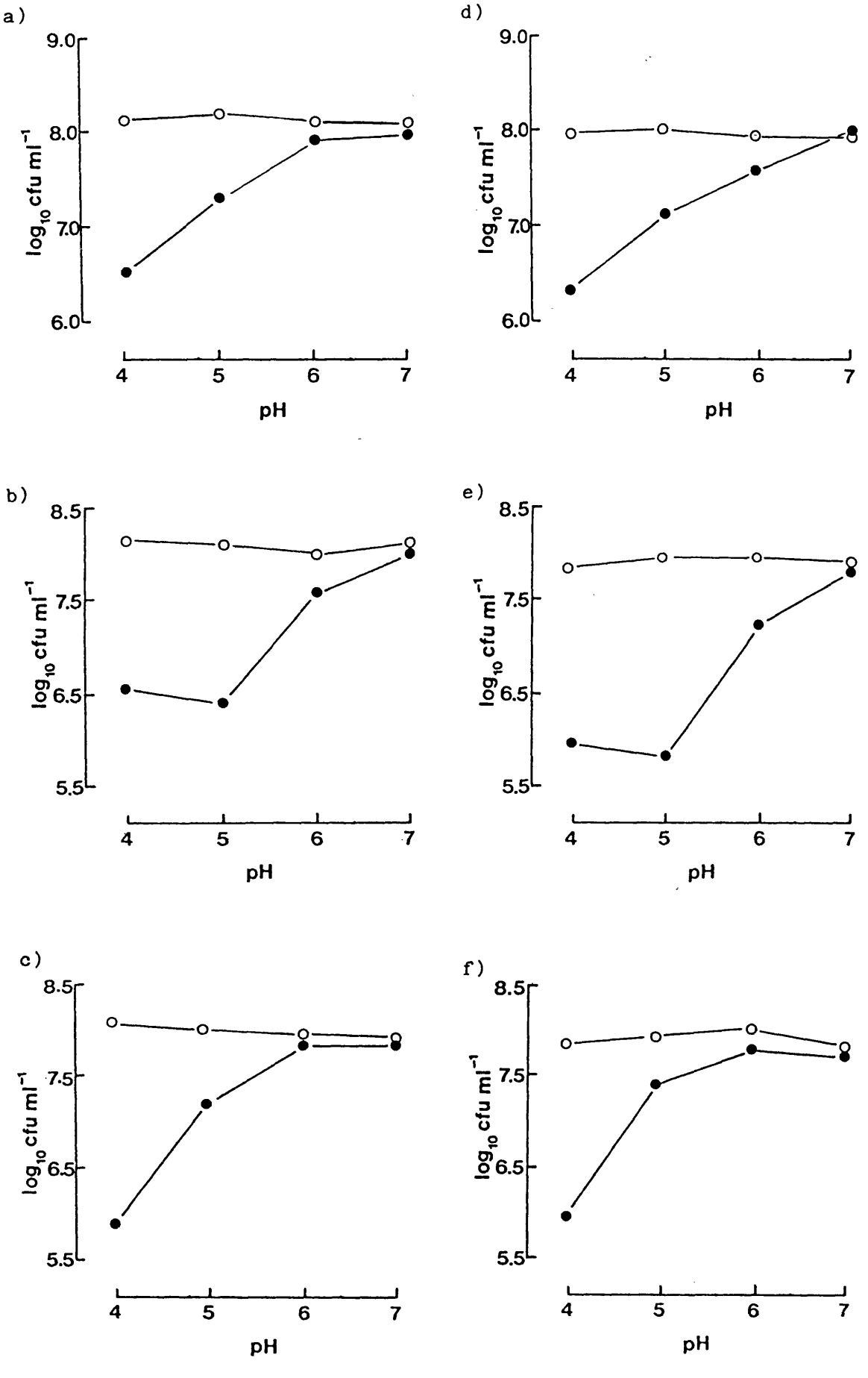
e) Lactate - Candida vini

f) Ethanol - Candida norvegica

○ Unsulphited

● Sulphited (500 µg SO_2 ml^{-1})

Points - mean of 5 observations


a)
9.0
8.0
7.0
6.0
log10 cfu ml-1
4
5
6
7
pH
d)
9.0
8.0
7.0
6.0
log10 cfu ml-1
4
5
6
7
pH
b)
8.5
7.5
6.5
5.5
log10 cfu ml-1
4
5
6
7
pH
e)
8.5
7.5
6.5
5.5
log10 cfu ml-1
4
5
6
7
pH
c)
8.5
7.5
6.5
5.5
log10 cfu ml-1
4
5
6
7
pH
f)
8.5
7.5
6.5
5.5
log10 cfu ml-1
4
5
6
7
pH

broth. Again, growth of C. norvegica at pH 4 was completely inhibited by sulphite, in fructose or ethanol broth, as was the case with glucose or lactate broth cultures (Figs. 37c,f).

d) The Effect of Inoculum Size

The effect of a larger initial cell density (ca 10^7 c.f.u.ml^{-1}) on sulphite tolerance of yeasts was investigated with lab lemco glucose or lactate broth over the pH range 4-7. The growth of C. norvegica and C. vini was unaffected by sulphite at pH 7 or 6 (Figs. 38, 39) but was completely inhibited at pH 4 and that of the latter at pH 5 (Figs. 38, 39) as was the case with a smaller initial cell density (ca 10^5 c.f.u.ml^{-1}). Sulphite inhibition of C. norvegica at pH 5, however, was notably reduced with the larger inoculum as compared to the extent of inhibition with the smaller inoculum (Figs. 34b, 36b).

e) The Effect of the Origin of Inoculum

In the experiments discussed above, inoculum was always taken from a broth culture containing the same substrate (i.e. glucose or lactate) as the experimental flask, as it was noted that the growth of C. norvegica, in unsulphited lab lemco glucose or lactate broth (pH 4, 5, 6 or 7) was slightly reduced when the inoculum originated from an unsupplemented lab lemco broth culture. The extent of growth inhibition by sulphite, however, was comparable at pH 7, 6 or 4 with inoculum from unsupplemented or supplemented (glucose or lactate) broth. In contrast, sulphite completely inhibited the growth of C. norvegica in lab lemco glucose, fructose, lactate or

Figure 38. The effect of pH on the growth of yeasts in lab lemco glucose broth with an initial density of ca 10^7 c.f.u.ml^{-1}, incubated at 25°C.

Candida norvegica

a) pH 4

b) pH 5

c) pH 6

d) pH 7

Candida vini

e) pH 4

f) pH 5

g) pH 6

h) pH 7

○ Unsulphited

● Sulphited (500 µg SO_2 ml^{-1})

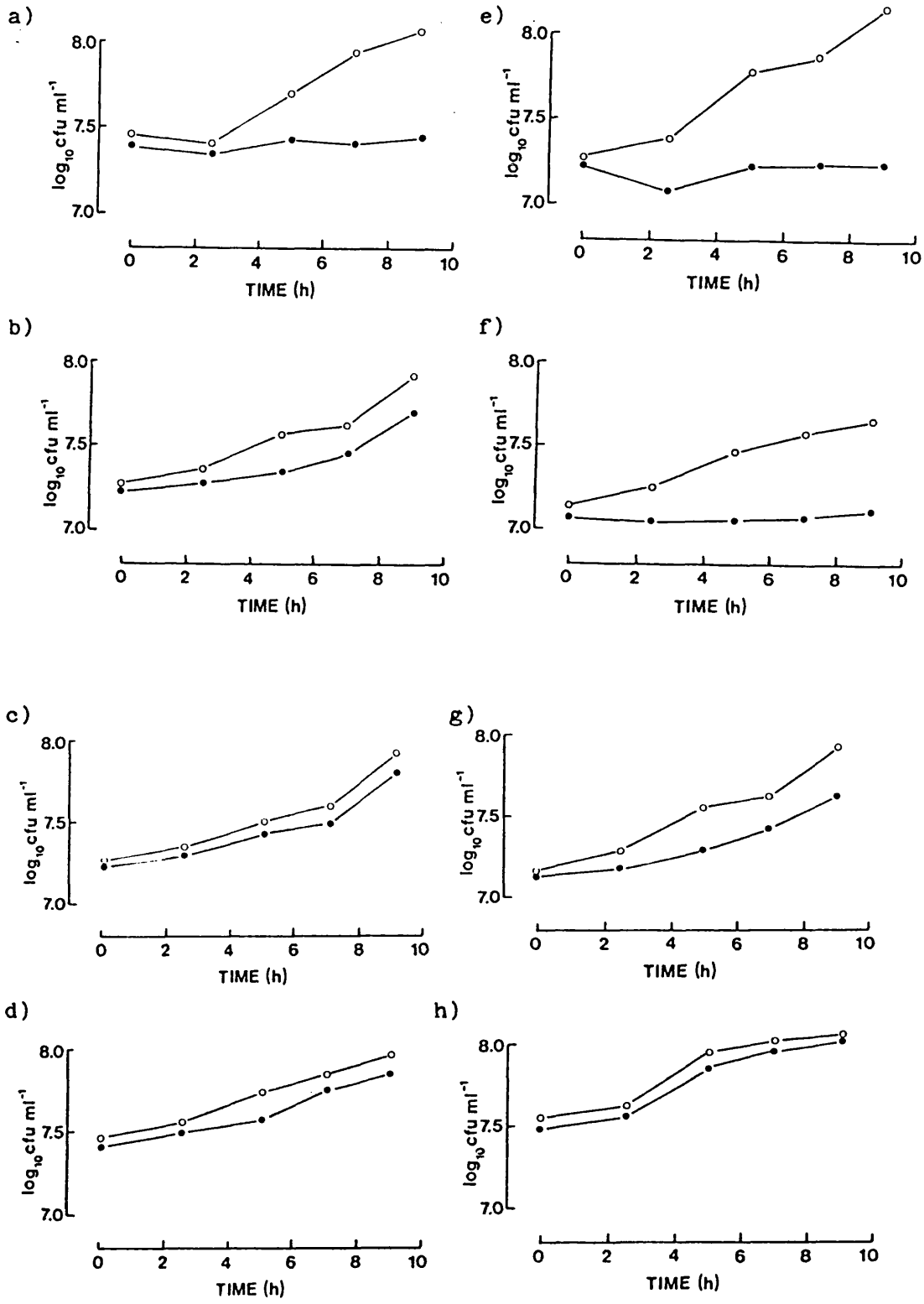

a)
e)
b)
f)
c)
g)
d)
h)
8.0
7.5
7.0
log10 cfu ml-1
0
2
4
6
8
10
TIME (h)

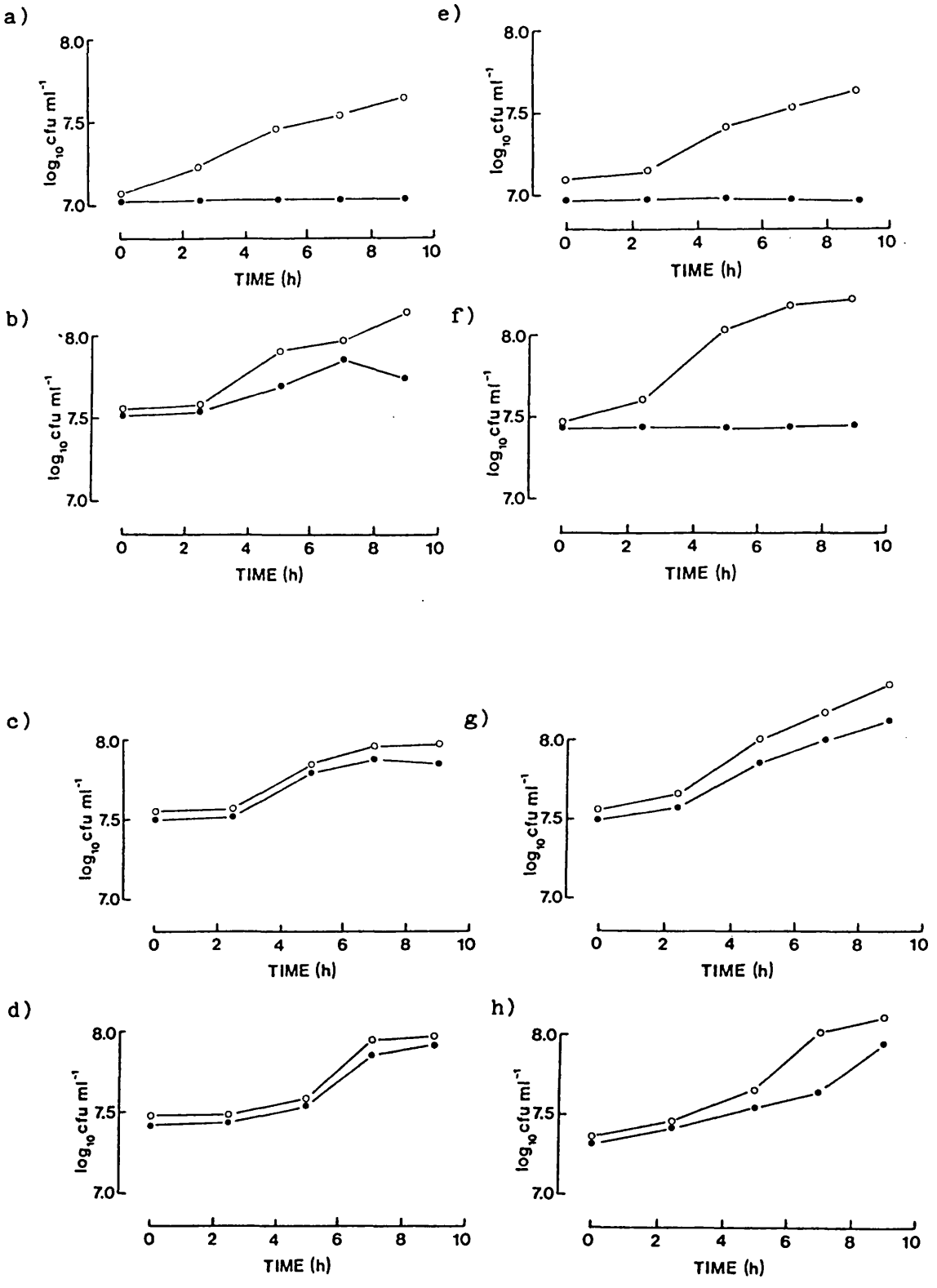
Figure 39. The effect of pH on the growth of yeasts in lab lemco lactate broth with an initial density of ca 10^7 c.f.u. ml^{-1}, incubated at 25°C.

Candida norvegica
a) pH 4
b) pH 5
c) pH 6
d) pH 7

Candida vini
e) pH 4
f) pH 5
g) pH 6
h) pH 7

○ Unsulphited

● Sulphited (500 µg SO_2 ml^{-1})


a)
b)
c)
d)
e)
f)
g)
h)
8.0
7.5
7.0
log10 cfu ml-1
0
2
4
6
8
10
TIME (h)

ethanol broth buffered at pH 5 when the inoculum originated from an unsupplemented lab lemco broth culture as compared to that from a glucose, fructose, lactate or ethanol broth (Figs. 40a, b, 41).

Growth inhibition of C. norvegica by sulphite at pH 5, when the inoculum originated from unsupplemented lab lemco broth could be associated with a lower level of carbohydrate reserve in the cells. The total carbohydrate content of cells of C. norvegica that originated from unsupplemented lab lemco broth was 27% of the dry cell weight as compared to 39% in cells derived from a glucose broth culture (Table 31). The low molecular weight fraction (including monosaccharides and disaccharides) accounted for 6.6% of the former and 11.9% of the latter (Table 31). The high molecular weight fraction (including polysaccharides) accounted for 19.8% of the dry cell weight from an unsupplemented lab lemco broth and 35.2% of the dry cell weight from a glucose broth culture.

SULPHITE BINDING

a) The Effect of pH

Candida vini was negative on the acetaldehyde detection medium and was therefore a non-sulphite binding yeast. This observation was confirmed by studies with sulphited (500 µg SO_2 ml^{-1}) broth cultures. The amount of sulphite bound in lab lemco glucose or lactate broth cultures of C. vini at pH 4, 5, 6 or 7 was not significantly different from the level of bound sulphite in the uninoculated controls (Table 28, Figs. 42, 43, 44,45b,e) and negligible quantities of acetaldehyde were recovered. It is noteworthy that the growth of C. vini was completely inhibited by

Figure 40. The effect of the pre-incubation medium on the growth of Candida norvegica in lab lemco broth (pH 5) incubated at 25°C for 20 h.

a) Unsulphited broth

b) Sulphited broth (500 µg SO_2 ml^{-1})

LL Lab lemco

L Lactate

F Fructose

G Glucose

Average of 2 observations

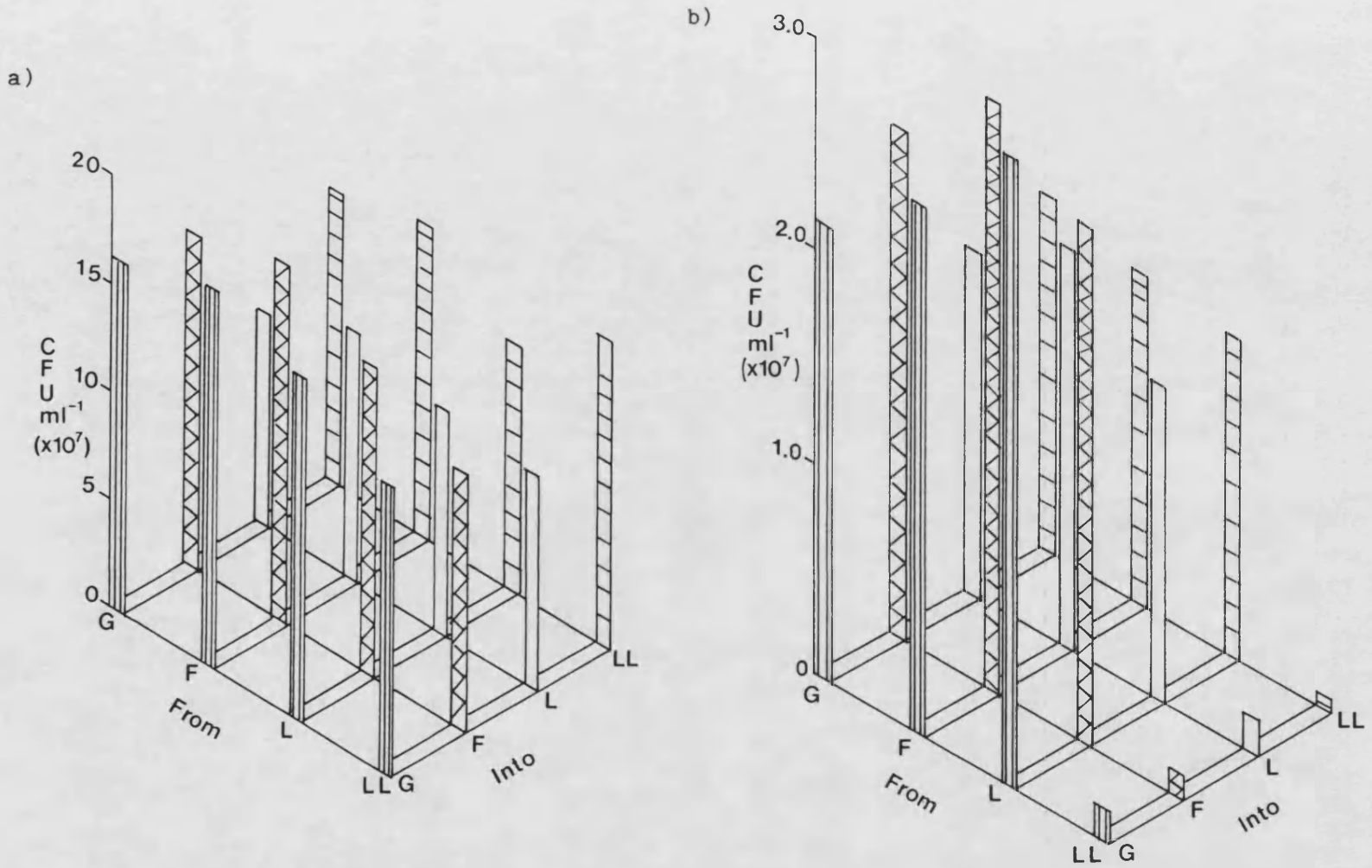

a)
20
15
10
5
0
CFU ml⁻¹ (x10⁷)
G
F
L
LL
From
G
F
L
LL
Into
b)
3.0
2.0
1.0
0
CFU ml⁻¹ (x10⁷)
G
F
L
LL
From
G
F
L
LL
Into

Figure 41. The effect of the pre-incubation medium on the growth of Candida norvegica in lab lemco ethanol broth (pH 5) incubated at 25°C for 20 h.

E Inoculum from ethanol broth

G Inoculum from glucose broth

LL Inoculum from lab lemco broth

Unsulphited

Sulphited (500 µg SO_2 ml^{-1})

Average of 3 observations

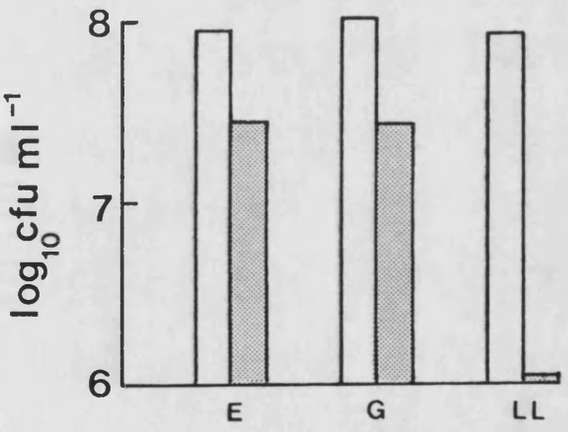

8
7
6
log10 cfu ml-1
E
G
LL

Table 31. Total carbohydrates in cells of Candida norvegica incubated in lab lemco and glucose broths.

Incubation medium	µg ml^{-1} (glucose equivalent)	total carbohydrate as % of dry weight
LL*		
Intact cells	51.4	27.0
TCA Extract	12.6	6.6
Pellet	37.7	19.8
G		
Intact cells	74.4	39.2
TCA Extract	22.6	11.9
Pellet	66.9	35.2

* LL = Lab lemco broth; G = Glucose broth

A yeast suspension of a standardised density equivalent to the dry weight of 190 µg ml^{-1} was used throughout.

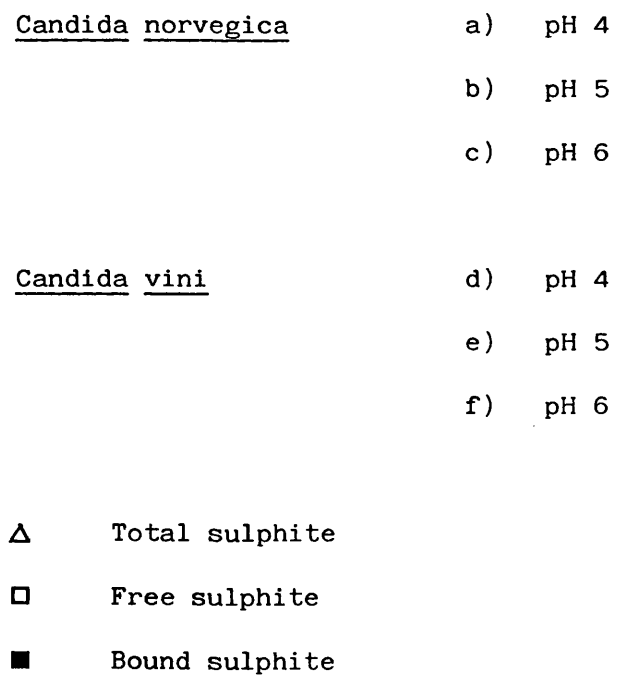
sulphite (500 µg SO_2 ml^{-1}) at pH 4 and 5 (Figs. 37b,e) but was unaffected at pH 6 and 7 (Figs. 37b,e).

Sulphite binding yeasts did not produce acetaldehyde when lactate was substituted for glucose in the acetaldehyde detection medium. Again this observation was confirmed by studies with sulphited (500 µg SO_2 ml^{-1}) broth cultures. The level of sulphite bound in lab lemco lactate broth cultures of C. norvegica at pH 4, 5, 6 or 7 was not significantly different from the amount of bound sulphite in the uninoculated controls (Table 28, Fig. 42) or in C. vini cultures (Table 30b). Thus only negligible quantities of acetaldehyde were recovered. Growth was completely inhibited at pH 4, reduced at pH 5 and 6 but unaffected at pH 7 by sulphite (500 µg SO_2 ml^{-1}) although acetaldehyde was not produced and sulphite was not bound (Fig. 42, 45d).

In lab lemco glucose broth cultures of C. norvegica there was a small loss of total sulphite with time, but a major loss of free sulphite associated with an increase in bound sulphite and acetaldehyde. The amount of sulphite bound, however, was pH dependent. Each specified pH value was maintained throughout incubation with all inoculated and uninoculated broths.

At pH 4 the level of bound sulphite was not significantly different from that in the uninoculated broth (Table 28). This reflected the fact that C. norvegica was not growing and therefore was not producing acetaldehyde. In contrast, the amount of sulphite bound at pH 7, 6 and 5 was significantly greater than in the uninoculated broths (Table 28).

Figure 42. The effect of pH on sulphite binding and acetaldehyde production by yeasts in lab lemco lactate broth incubated at 25°C:



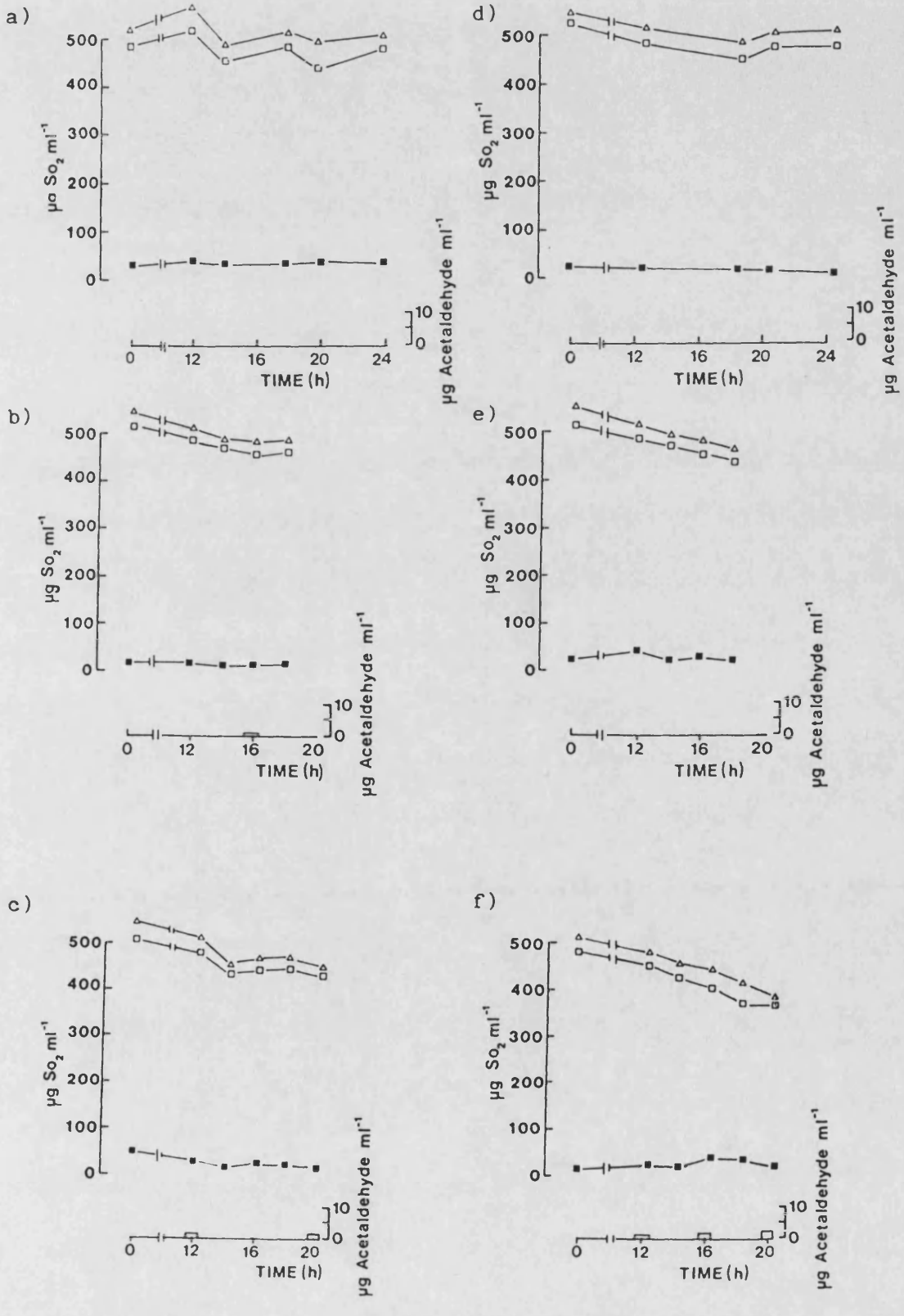

a)
b)
c)
d)
e)
f)
µg So_2 ml^{-1}
µg Acetaldehyde ml^{-1}
TIME (h)
0
100
200
300
400
500
10
12
16
20
24

Figure 43. The effect of pH on sulphite binding and acetaldehyde production by yeasts in lab lemco glucose broth incubated at 25°C:

Candida norvegica	a)	pH 4
	b)	pH 5
	c)	pH 6
Candida vini	d)	pH 4
	e)	pH 5
	f)	pH 6

△ Total sulphite

□ ○ Free sulphite

■ ● Bound sulphite

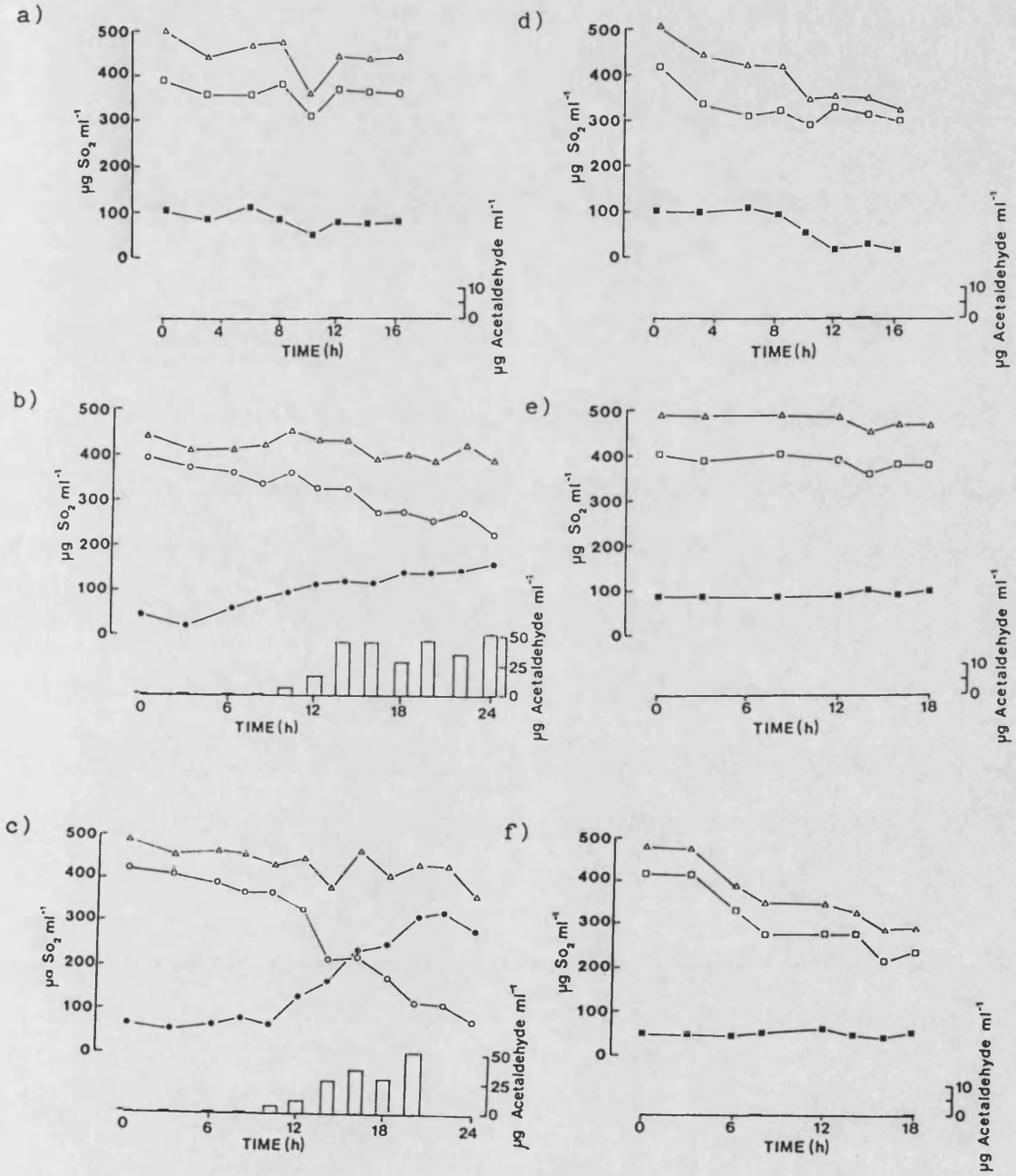

a)
b)
c)
d)
e)
f)
µg So_2 ml^{-1}
µg Acetaldehyde ml^{-1}
TIME (h)

Figure 44. The effect of pH on sulphite binding and acetaldehyde production by yeasts in lab lemco glucose broth incubated at 25°C:

Unbuffered broth initially poised at pH 7 reduced to pH 5.6 after 24 h.

a) Candida norvegica

b) Candida vini

Broth buffered at pH 7

c) Candida norvegica

d) Candida vini

△ Total sulphite

□ Free sulphite

■ Bound sulphite

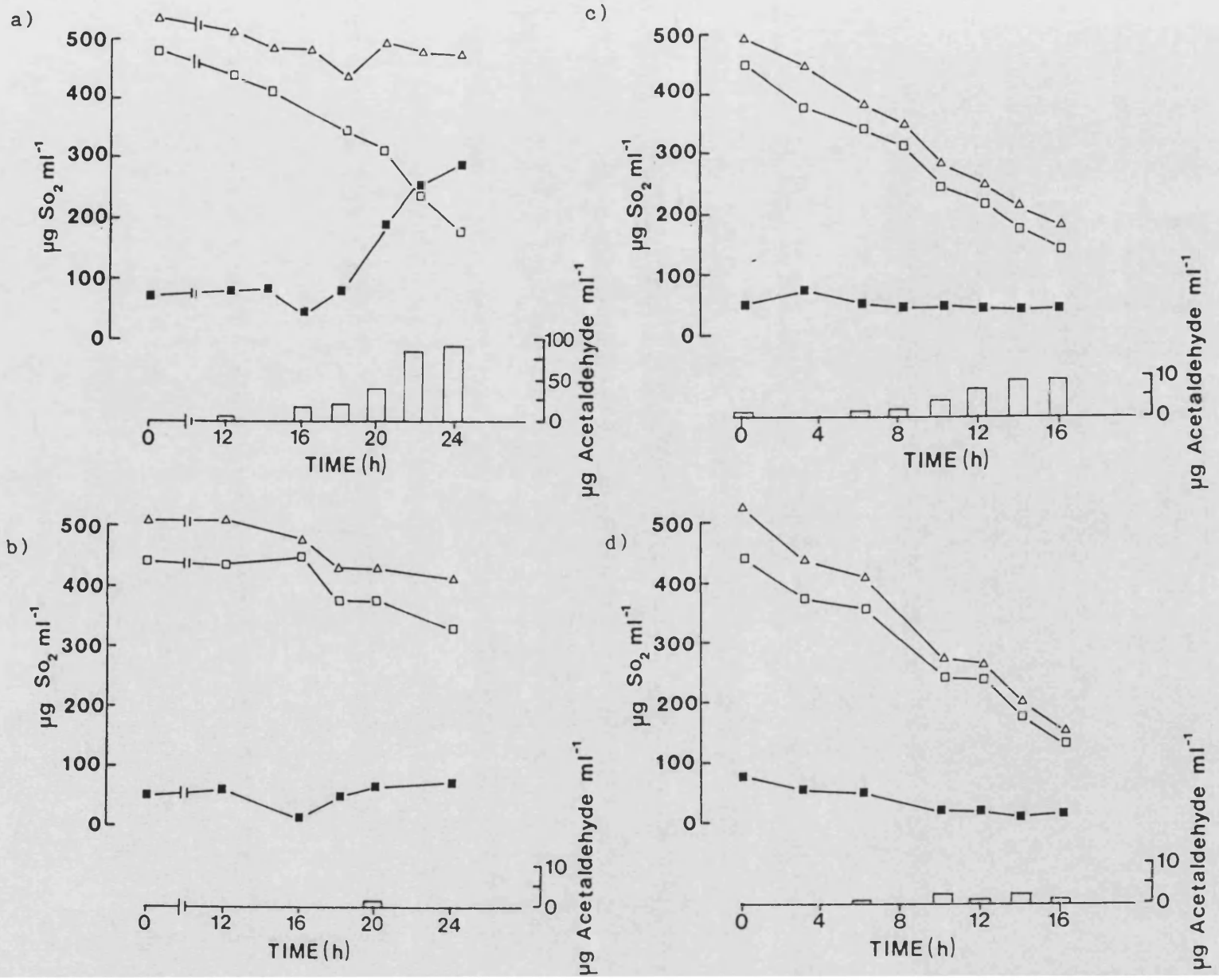

a)
b)
c)
d)
µg So_2 ml^{-1}
µg Acetaldehyde ml^{-1}
TIME (h)
0
100
200
300
400
500
50
4
8
12
16
20
24

Figure 45. The effect of pH on sulphite binding and acetaldehyde production by yeasts in lab lemco broth incubated at 25°C for 20 h.

a) Glucose - *Candida norvegica*

b) Glucose - *Candida vini*

c) Fructose - *Candida norvegica*

d) Lactate - *Candida norvegica*

e) Lactate - *Candida vini*

f) Ethanol - *Candida norvegica*

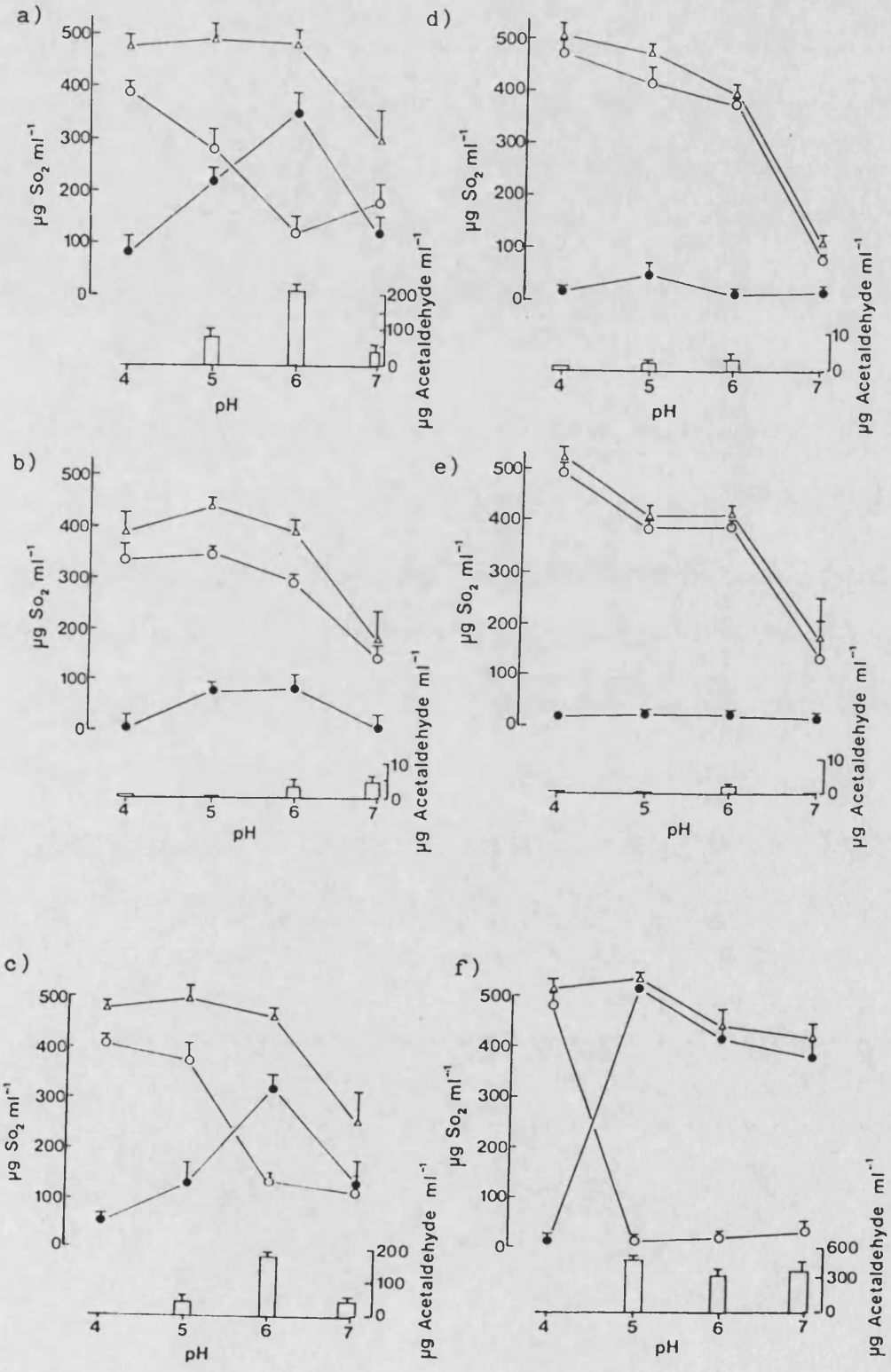
△ Total sulphite

○ Free sulphite

● Bound sulphite

I Standard Error Bars

Points - mean of 5 observations


a)
b)
c)
d)
e)
f)
µg So_2 ml^{-1}
µg Acetaldehyde ml^{-1}
pH
0
100
200
300
400
500
4
5
6
7
10
600

At pH 7 sulphite was rapidly lost during incubation but only a minor proportion of this loss was recovered as bound sulphite (Fig. 44). A qualitative test indicated that sulphite was being irretrievably lost by oxidation to sulphate in inoculated and uninoculated broths. Consequently, the low amount of bound sulphite in cultures of C. norvegica (123 μg SO_2 ml^{-1}), accounted for 41% of the total available sulphite (300 μg SO_2 ml^{-1}) - Table 32. The acetaldehyde concentration (40 μg ml^{-1}) was also low (Table 32).

The amount of sulphite bound at pH 5 was significantly greater than that at pH 4 or 7 (Table 26). An initial interval of 3 h before suphite binding commenced corresponded with the lag phase of growth of C. norvegica (Figs. 34b, 43b). The subsequent gradual increase in bound suphite and concomitant increase in acetaldehyde concentration (Fig. 43b) was proportional to the slow rate of cell division occurring during the exponential phase of growth (Fig. 34b, Table 23).

At pH 6 the amount of bound sulphite was significantly greater than that bound at pH 5 (Table 26, Fig. 45a). During the exponential phase of growth of C. norvegica there was a proportional increase in bound sulphite (Fig. 43c) so that by 20 h (stationary phase) ca 75% was bound and no further increase in bound sulphite occurred. The concentration of acetaldehyde (207 μg ml^{-1}) at pH 6 was significantly greater than that at pH 5 or 7 (Table 24, 26). Assuming that the binding of acetaldehyde to sulphite was 1:1 (Burroughs and Sparks, 1973a), the acetaldehyde concentration at pH 6 was sufficient to bind 63% of the sulphite available (Table 32). Glucose (0.1 - 2% w/v) was required at pH 6

Table 32. The influence of substrates and pH on sulphite binding by yeasts with incubation at 25°C for 20 h.

pH	Substrate	Acetaldehyde[1] µg ml^{-1} (% sulphite bound[3])	% Bound[2] sulphite
Candida norvegica			
4	glucose	0.46 (0.14)	20.36
5	glucose	77.60 (22.85)	42.91
6	glucose	207.10 (62.75)	74.60
7	glucose	40.40 (19.76)	41.36
4	fructose	0.09 (0.03)	12.95
5	fructose	45.00 (12.84)	26.40
6	fructose	175.30 (54.65)	69.90
7	fructose	45.30 (25.84)	52.03
4	lactate	1.07 (0.30)	4.70
5	lactate	2.50 (0.75)	12.15
6	lactate	2.80 (1.00)	6.00
7	lactate	0.19 (0.24)	21.89
4	ethanol	0 (0)	4.27
5	ethanol	504.80 (137.55)	98.65
6	ethanol	352.45 (114.84)	95.34
7	ethanol	407.16 (144.21)	92.77
Candida vini			
4	glucose	0.09 (0.03)	11.67
5	glucose	0.28 (0.09)	21.02
6	glucose	4.51 (1.62)	24.23
7	glucose	5.10 (3.86)	16.49
4	lactate	0.29 (0.08)	3.77
5	lactate	0.09 (0.03)	4.99
6	lactate	1.79 (0.64)	6.79
7	lactate	0.19 (0.16)	17.31

1. Determined by Boehringer Mannheim assay kit

2. Determined by the method of Banks and Board (1982a)

3. Determined by assuming a 1:1 acetaldehyde : sulphite ratio (Burroughs and Sparks, 1973a).

for acetaldehyde production, sulphite binding, and to maintain the total sulphite concentration (Fig. 46).

The unbuffered lab lemco glucose broth culture of C. norvegica was initially pH 7 but diminished to pH 5.6 after 24 h incubation at 25°C. Candida norvegica grew for 16 h before any sulphite was bound (Fig. 35a) as was the case with the pH 7 broth culture. A subsequent increase in bound sulphite occurred and by 24 h 62% was bound (Fig. 44a). The latter reflected the results obtained with the pH 6 broth indicating that binding occurred in response to an acid drift.

b) The Effect of Sulphite Concentration

Candida vini (a confirmed non-sulphite binder) did not produce acetaldehyde or bind sulphite in lab lemco glucose broth buffered at pH 4-7 and containing 100-1000 μg SO_2 ml^{-1} (Figs. 45, 47, 48). Indeed the amount of sulphite bound in cultures of C. vini was comparable to that bound in the uninoculated controls. Growth was noted, however, to be completely inhibited by 100 μg SO_2 ml^{-1} at pH 4 and 500 μg SO_2 ml^{-1} at pH 5 (Fig. 31).

The amount of sulphite binding and acetaldehyde produced in cultures of C. norvegica was dependent on pH and sulphite concentration (Figs. 45, 47, 48). Thus at pH 4 with 100 μg SO_2 ml^{-1}, growth of C. norvegica was associated with sulphite binding (91%). At greater concentrations of sulphite (250 μg SO_2 ml^{-1}) growth was completely inhibited (Fig. 31) and the amount of bound sulphite (22%) was comparable to that bound in the uninoculated

Figure 46. The effect of glucose concentration at pH 6 on sulphite binding and acetaldehyde production by Candida norvegica incubated at 25°C for 20 h.

△ Total sulphite

○ Free sulphite

● Bound sulphite

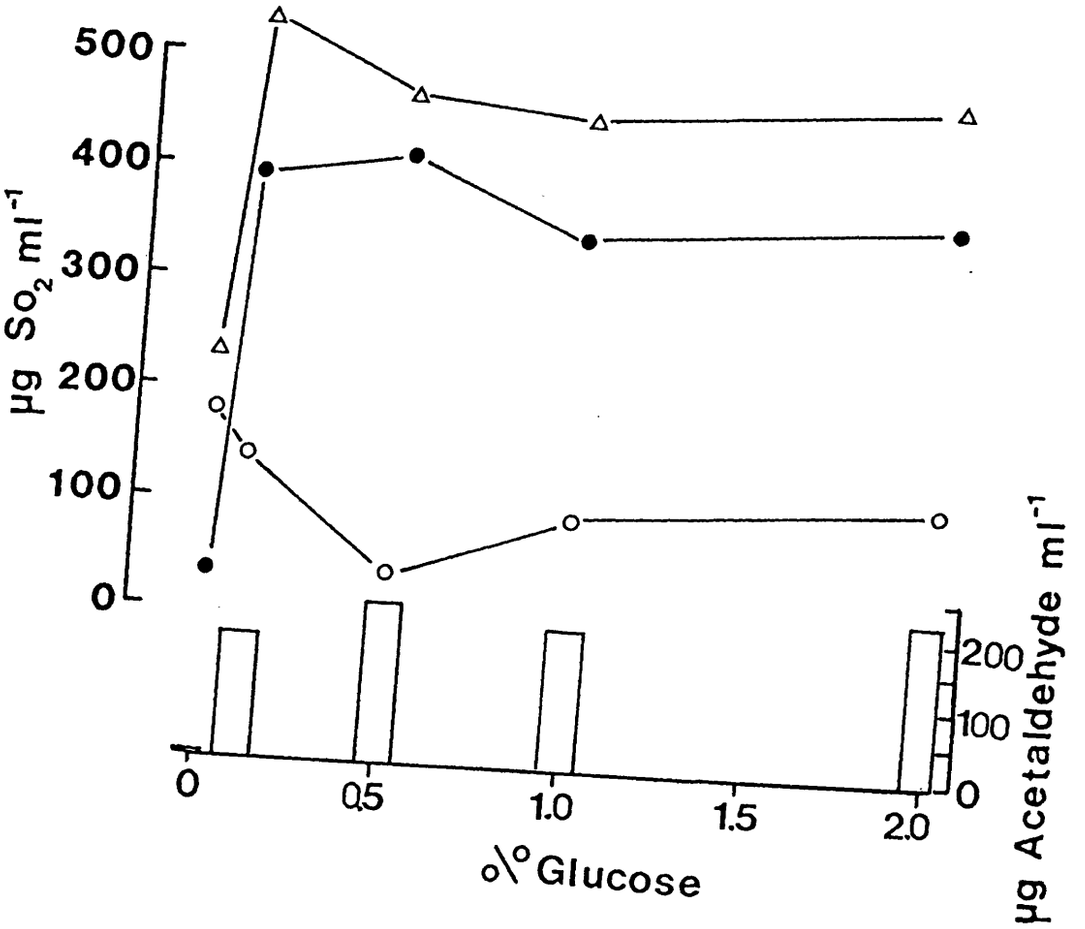

500
400
300
200
100
0
µg So_2 ml^{-1}
0
0.5
1.0
1.5
2.0
%° Glucose
200
100
0
µg Acetaldehyde ml^{-1}

Figure 47. The effect of sulphite concentration on sulphite binding and acetaldehyde production by yeasts incubated in lab lemco glucose broth at 25°C for 20 h.

a) *Candida norvegica* - 100 µg SO_2 ml^{-1}

b) *Candida vini* - 100 µg SO_2 ml^{-1}

c) *Candida norvegica* - 250 µg SO_2 ml^{-1}

d) *Candida vini* - 250 µg SO_2 ml^{-1}

△ Total sulphite

□ Free sulphite

■ Bound sulphite

a)
µg So2 ml-1
0
50
100
pH
4
5
6
7
0
20
µg Acetaldehyde ml-1
b)
µg So2 ml-1
0
50
100
pH
4
5
6
7
0
20
µg Acetaldehyde ml-1
c)
µg So2 ml-1
0
100
200
300
pH
4
5
6
7
0
60
µg Acetaldehyde ml-1
d)
µg So2 ml-1
0
100
200
300
pH
4
5
6
7
0
30
µg Acetaldehyde ml-1

Figure 48. The effect of sulphite concentration on sulphite binding and acetaldehyde production by yeasts incubated in lab lemco glucose broth at 25°C for 20 h.

a) *Candida norvgica* - 750 µg SO_2 ml^{-1}

b) *Candida vini* - 750 µg SO_2 ml^{-1}

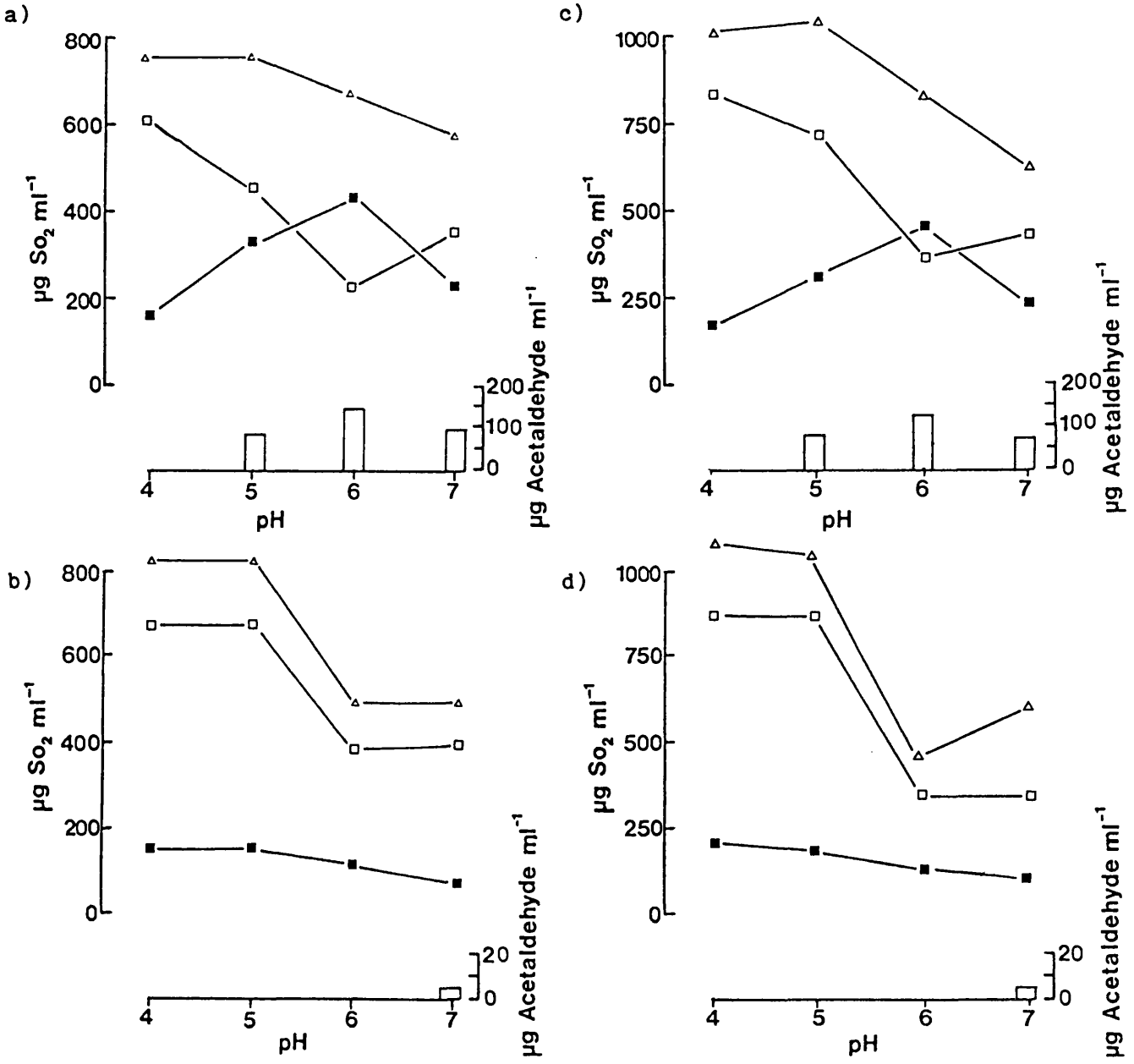
c) *Candida norvegica* - 1000 µg SO_2 ml^{-1}

d) *Candida vini* - 1000 µg SO_2 ml^{-1}

△ Total sulphite

□ Free sulphite

■ Bound sulphite


a)
b)
c)
d)
µg So_2 ml^{-1}
µg Acetaldehyde ml^{-1}
pH
0
200
400
600
800
250
500
750
1000
100
20
4
5
6
7

broths.

In lab lemco glucose broth cultures of C. norvegica buffered at pH 5, 96% of the sulphite was bound at 100 and 250 μg SO_2 ml^{-1} (Figs. 45, 47, 48). At greater concentrations of sulphite the amount of sulphite bound did not increase proportionally with the increase in total sulphite (Figs. 45, 47, 48).

In lab lemco glucose broth cultures of C. norvegica buffered at pH 6, 70% of the sulphite was bound with 100 μg SO_2 ml^{-1} (Fig. 47a). At 500-1000 μg SO_2 ml^{-1} a similar quantity of sulphite was bound but accounted for a decreasing percentage of the total sulphite (Figs. 45, 47, 48). At pH 7,64% of the sulphite was bound at 100 and 250 μg SO_2 ml^{-1}, but at higher concentrations a large proportion of the sulphite was irretrievably lost presumably by oxidation to sulphate (Figs. 45, 47, 48).

c) The Effect of Substrate

Sulphite binding by C. norvegica was investigated further with a survey of substrates, many of which occur in meat. The percentage of bound sulphite in lab lemco (pH 6) broth cultures of C. norvegica supplemented with (0.5-2% w/v): acetate, cellobiose, glycerol, 3-O-methyl glucose, lactate, maltose, salicin, sorbitol, sorbose, succinate, sucrose or starch was not significantly different to the amount of sulphite bound in the uninoculated broths (Table 33). Similarly, the amount of sulphite irretrievably lost (20-80%), presumably by oxidation to sulphate, was comparable with the inoculated and uninoculated broths, for each substrate. The greatest percentage of sulphite was irretrievably lost with

Table 33. The influence of substrates at pH 6 on sulphite binding by *Candida norvegica* with incubation at 25°C for 20 h.

Substrate (0.5 or 1% w/v)	Acetaldehyde[1] µg ml^{-1} (% sulphite bound[3])	% Bound[2] sulphite	
Acetate	2.1 (2.6)	21.9	
cellobiose	0.4 (0.3)	9.7	
glycerol	1.0 (0.5)	7.3	
3-O-methyl glucose	1.3 (1.2)	20.1	
lactate	2.8 (1.0)	6.0	
maltose	3.8 (1.5)	7.6	
salicin	0.9 (0.4)	6.5	$p>0.1$[4]
sorbitol	2.7 (1.5)	10.5	
sorbose	3.0 (1.9)	13.7	
succinate	0.5 (0.6)	31.5	
sucrose	1.6 (0.8)	7.2	
starch	0.5 (0.6)	17.1	
pyruvate	0.03 (0.01)	(89.9)[6]	
fructose	175.3 (54.6)	69.9	
glucose	207.1 (62.7)	74.6	$p<0.001$[5]
ethanol	352.4 (114.8)	95.3	

1. Determined by Boehringer Mannheim assay kit.
2. Determined by the method of Banks and Board (1982a).
3. Determined by assuming a 1:1 acetaldehyde : sulphite ratio (Burroughs and Sparks, 1973a).
4. Comparison (in duplicate) of percent sulphite bound in uninoculated and inoculated broth containing the listed substrate, no significant difference.
5. With fructose, glucose and ethanol (5 replicates), there was a significant difference ($p<0.001$) between sulphite bound in the inoculated and uninoculated broths.
6. Pyruvate bound sulphite to an equal extent in both inoculated and uninoculated broth.

succinate and acetate. With pyruvate, an avid binding agent, 89.9% of the sulphite was immediately bound to the substrate and no acetaldehyde was produced. Thus substrates had a greater effect on sulphite loss than on growth of C. norvegica (Fig. 32).

The acetaldehyde produced with lab lemco (pH 6) fructose (175 μg ml^{-1}) and glucose (207 μg ml^{-1}) broth cultures of C. norvegica accounted for the majority of the sulphite bound (Table 33). The acetaldehyde concentration in the ethanol (pH 6) broth cultures of C. norvegica (352.4 μg ml^{-1}), however, was in excess of that required to bind the sulphite (Table 33).

Sulphite binding by C. norvegica was further tested with lab lemco ethanol and fructose broths buffered at pH 4-7 (Figs. 45,c,f). The level of sulphite bound in lab lemco ethanol or fructose (pH 4) broth cultures of C. norvegica was not significantly different from that in the uninoculated broths (Table 28). This reflected the fact that sulphite (500 μg SO_2 ml^{-1}) completely inhibited the growth of C. norvegica at pH 4 (Fig. 37f).

The amount of sulphite bound in lab lemco fructose broth cultures of C. norvegica at pH 5, 6 and 7 was not significantly different from that bound in glucose broth cultures (Table 27). Again, sulphite was irretrievably lost at pH7. A significantly greater amount of sulphite was bound at pH 6 than at pH 5 or 7 (Table 26, Fig. 45c) as was the case with glucose broth cultures. These results reflect the fact that growth of C. norvegica was reduced by sulphite at pH 5 (Fig. 37c). Thus, the acetaldehyde concentration at pH 6 was significantly more than that at pH 7 or 5 and accounted for most of the 70% of the sulphite bound (Tables 26,

33, Fig. 45c).

The amount of sulphite bound in lab lemco ethanol broth cultures of C. norvegica buffered at pH 5, 6 or 7 was significantly greater than that bound in glucose and fructose broth cultures (Table 27). Assuming the binding of acetaldehyde to sulphite is 1:1 (Burroughs and Sparks, 1973a) the acetaldehyde produced in lab lemco ethanol broth cultures of C. norvegica buffered at pH 5, 6 or 7 was in excess of that required to bind the total available sulphite (Table 33, Fig. 45f). The amount of sulphite bound at pH 5 (526.6 μg SO_2 ml^{-1}) was significantly greater (Tables 24, 26) than that bound at pH 6 (425.6 μg SO_2 ml^{-1}). This reflects the fact that in the case of ethanol, sulphite reduces the growth of C. norvegica at pH 6 (Fig. 37f). Less sulphite was bound at pH 7 as a proportion of the sulphite was irretrievably lost presumably by oxidation to sulphate (Tables 26, 33, Fig. 45f).

d) The Effect of Inoculum Size

An initial cell density of ca 10^7 c.f.u.ml^{-1} was used to investigate the sulphite binding capacity of C. norvegica and C. vini in lab lemco glucose and lactate broth cultures buffered at pH 4-7 (Figs. 49, 50, 51). Sulphite binding and acetaldehyde production did not occur with cultures of C. vini or with lab lemco lactate broth cultures of C. norvegica, as was the case with cultures of an initial cell density of ca 10^5 c.f.u.ml^{-1} (Figs. 42, 43, 44).

In lab lemco glucose broth cultures of C. norvegica buffered at pH 6, 75% of the sulphite was bound after 9 h (10^7 c.f.u.ml^{-1}) or

Figure 49. The effect of pH on sulphite binding and acetaldehyde production by yeasts in lab lemco glucose broth with an initial density of ca 10^7 c.f.u.ml^{-1}, incubated at 25°C.

Candida norvegica a) pH 4

c) pH 5

Candida vini b) pH 4

d) pH 5

△ Total sulphite

□ Free sulphite

■ Bound sulphite

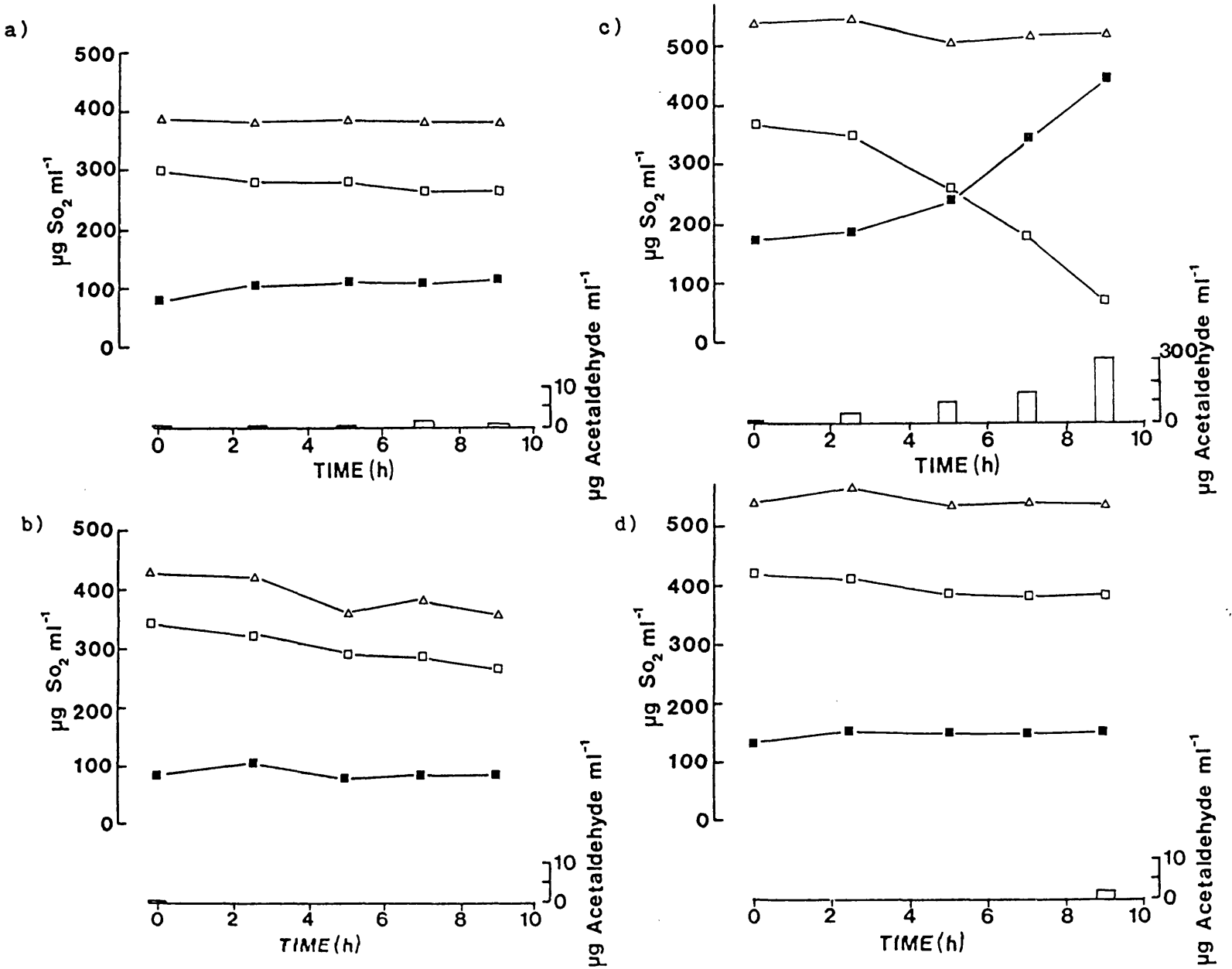

a)
b)
c)
d)
µg So_2 ml^{-1}
µg Acetaldehyde ml^{-1}
TIME (h)
0
100
200
300
400
500
2
4
6
8
10
300

Figure 50. The effect of pH on sulphite binding and acetaldehyde production by yeasts in lab lemco glucose broth with an initial density of ca 10^7 c.f.u.ml^{-1}, incubated at 25°C.

Candida norvegica a) pH 6

c) pH 7

Candida vini b) pH 6

d) pH 7

Δ Total sulphite

□ Free sulphite

■ Bound sulphite

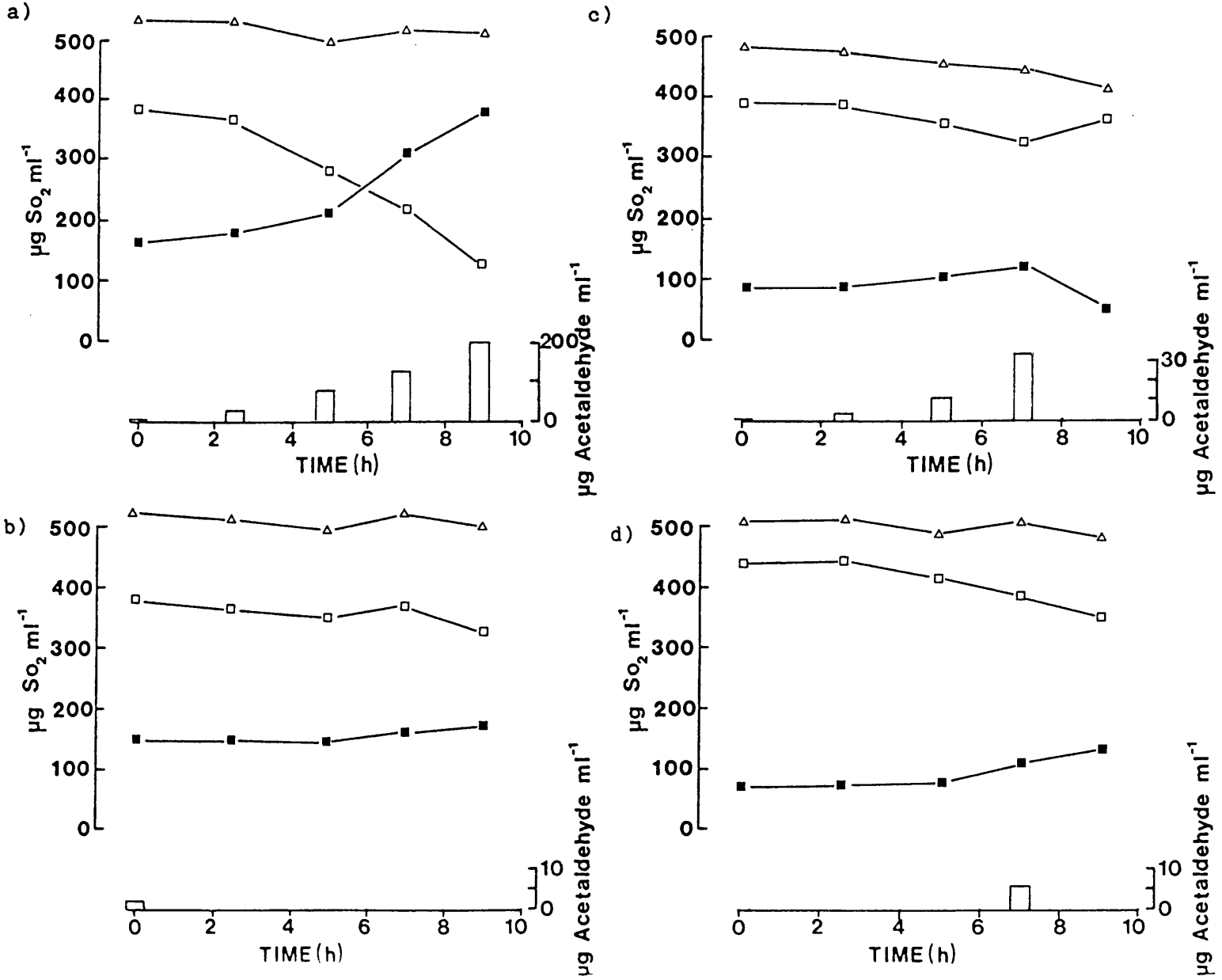
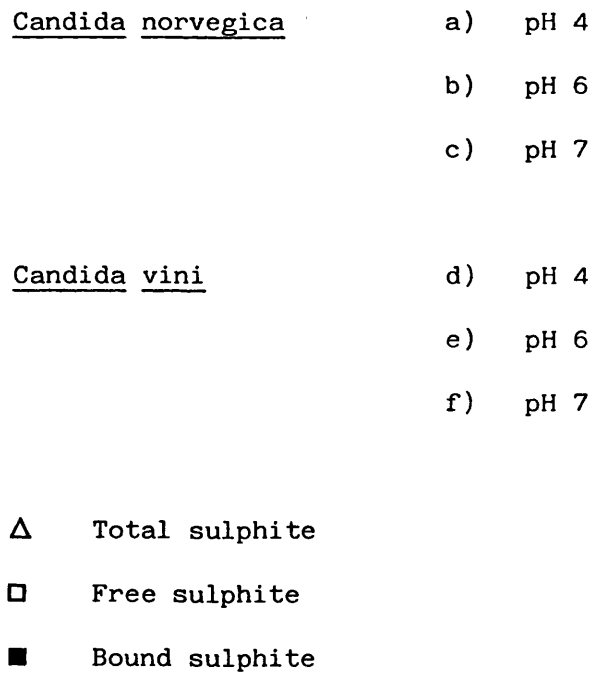
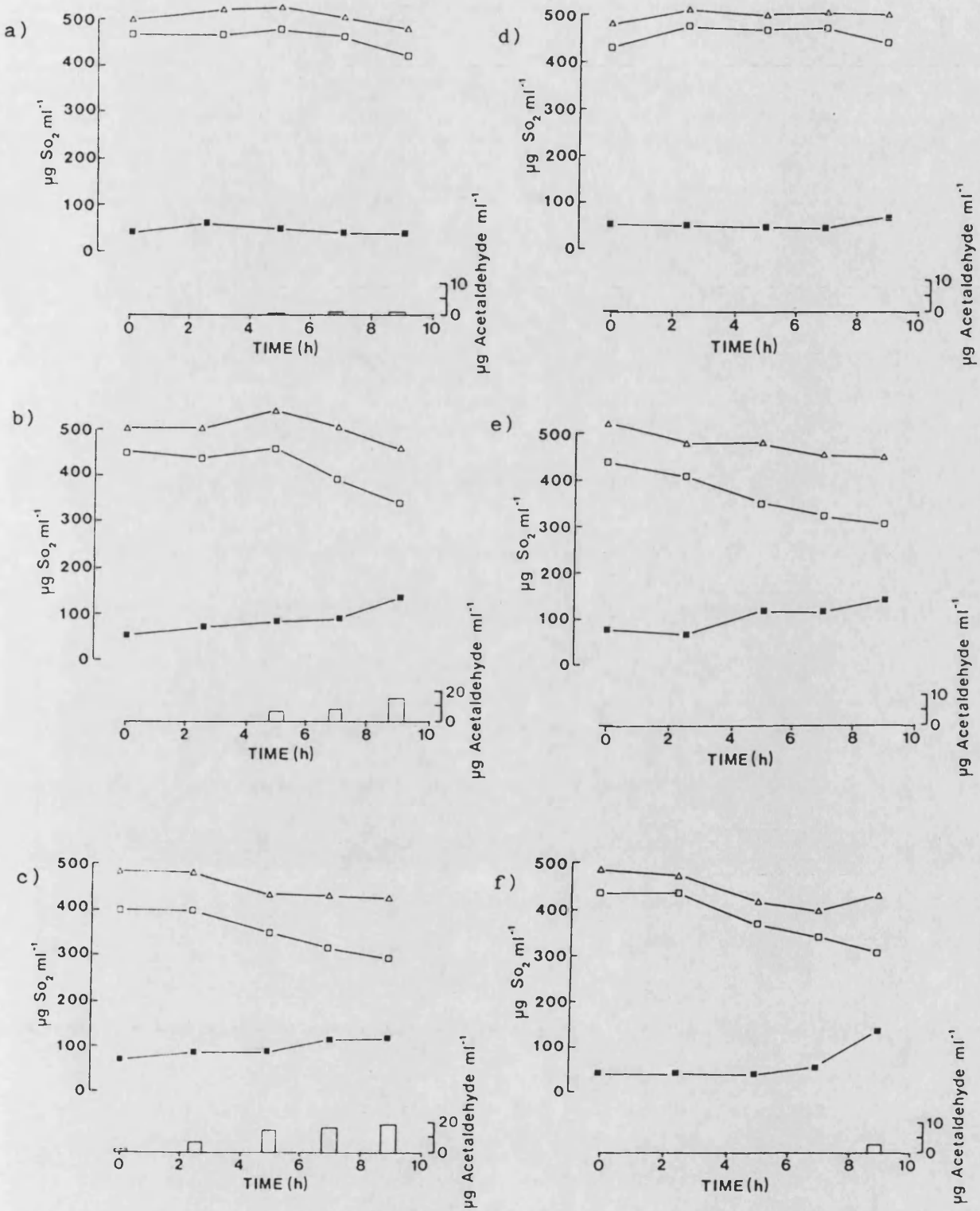

a)
b)
c)
d)
µg So_2 ml^{-1}
µg Acetaldehyde ml^{-1}
TIME (h)
0
100
200
300
400
500
2
4
6
8
10
30

Figure 51. The effect of pH on sulphite binding and acetaldehyde production by yeasts in lab lemco lactate broth with an initial density of ca 10^7 c.f.u.ml^{-1}, incubated at 25°C.




a)
b)
c)
d)
e)
f)
µg So_2 ml^{-1}
µg Acetaldehyde ml^{-1}
TIME (h)
0
100
200
300
400
500
2
4
6
8
10
20

20 h (10^5 c.f.u.ml^{-1}) and a similar amount of acetaldehyde was recovered (Figs. 43c, 50a). At pH 5 sulphite binding and acetaldehyde production was enhanced by the initial large cell density (Fig. 49c). Indeed, King et al. (1981) and Beech and Thomas (1985) noted that high cell densities contain larger concentrations of acetaldehyde thereby enhancing the sulphite tolerance of the yeast. No sulphite binding occurred at pH 4 as growth was completely inhibited (Fig. 49a) and only a little sulphite binding occurred at pH 7 (Fig. 50c).

e) The Effect of Origin of Inoculum

In lab lemco glucose, fructose or ethanol (pH 5) broth cultures of C. norvegica, acetaldehyde was produced and sulphite was bound only when the inoculum originated from a supplemented (fructose, glucose, lactate or ethanol) broth (Figs. 52, 53). This effect was pronounced in sulphited lab lemco ethanol broth cultures of C. norvegica. Acetaldehyde was produced in excess of that required to bind the total available sulphite when the inoculum was from glucose or ethanol broth and more than 510 μg ml^{-1} sulphite was bound (Fig. 53). In contrast, 15.5 μg ml^{-1} acetaldehyde was produced and 46.5 μg ml^{-1} sulphite bound (an amount comparable to that in the uninoculated broth) when the inoculum originated from unsupplemented lab lemco broth (Fig. 53). This reflected the fact that the growth of C. norvegica was completely inhibited when the inoculum was from an unsupplemented lab lemco broth culture (Fig. 41). The amount of sulphite bound in lactate or lab lemco (pH 5) broth cultures of C. norvegica was comparable to that bound

Figure 52. The effect of the pre-incubation medium on sulphite binding by Candida norvegica incubated at 25°C for 20 h.

a) The amount of bound sulphite

b) The amount of acetaldehyde produced

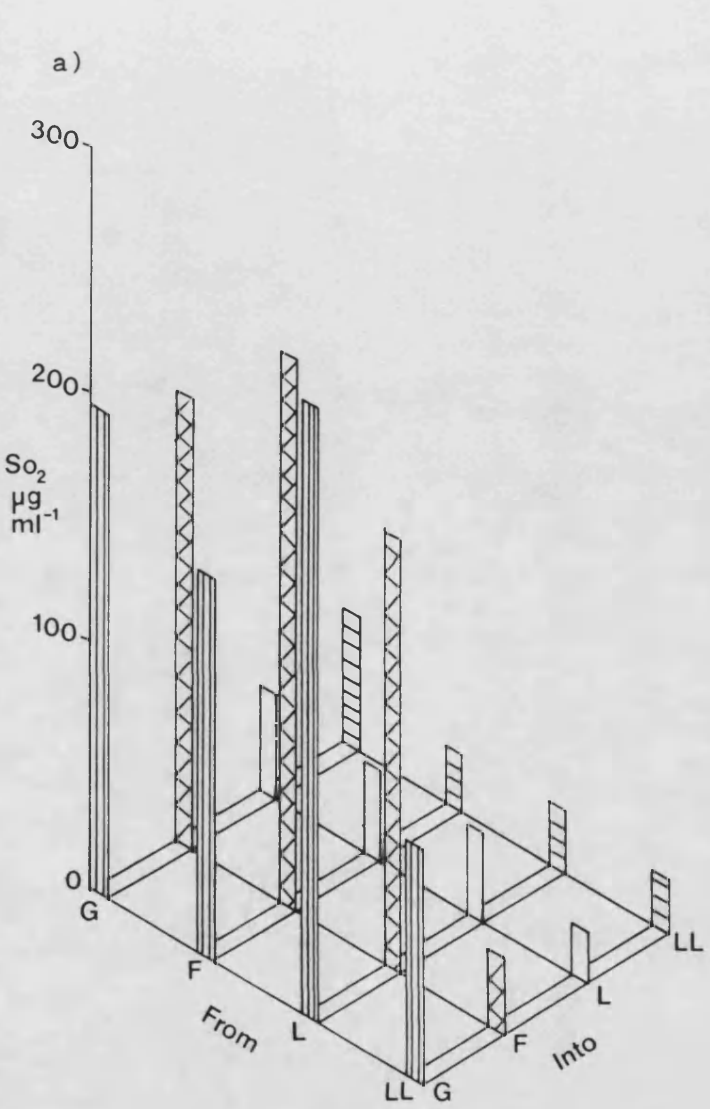
LL Lab lemco

L Lactate

F Fructose

G Glucose

Average of 2 observations


a)
300
200
100
0
So_2 µg ml^{-1}
G
F
L
LL
From
G
F
L
LL
Into

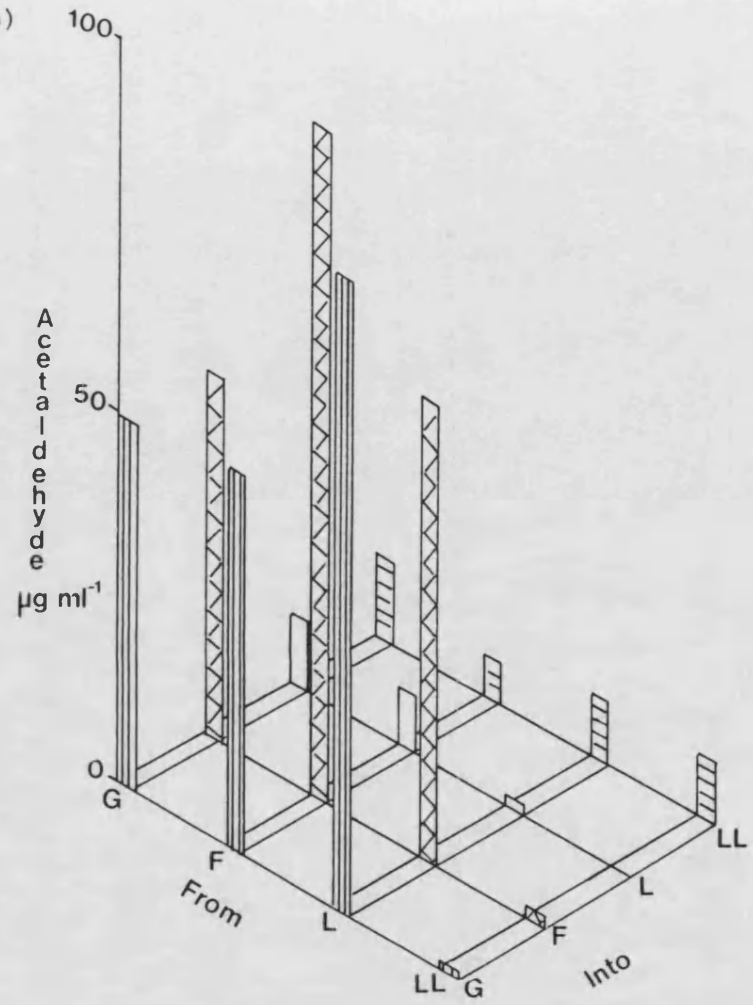

b)
100
50
0
Acetaldehyde µg ml^{-1}
G
F
L
LL
From
G
F
L
LL
Into

Figure 53. The effect of pre-incubation medium on sulphite binding and acetaldehyde production by Candida norvegica in lab lemco ethanol broth incubated at 25°C for 20 h.

a) sulphite binding

b) acetaldehyde production

□ Free sulphite

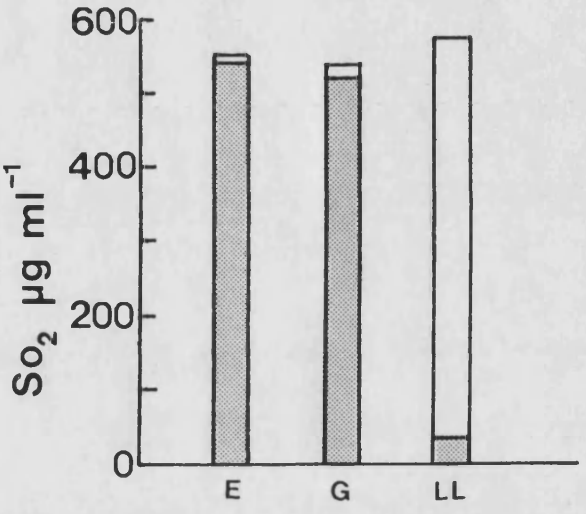
▒ Bound sulphite

E Inoculum from ethanol broth

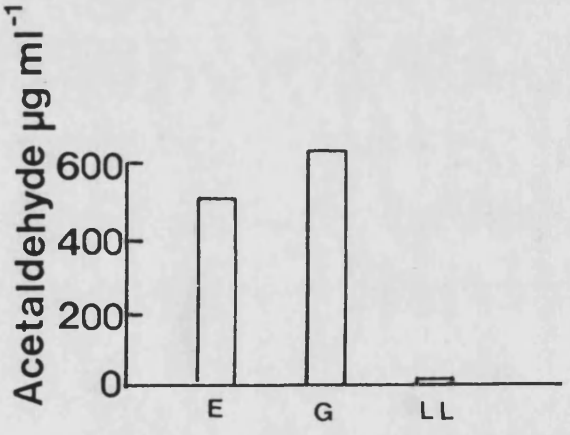
G Inoculum from glucose broth

LL Inoculum from lab lemco broth

a)


600
400
200
0
So_2 µg ml^{-1}
E
G
LL

b)


Acetaldehyde µg ml^{-1}
600
400
200
0
E
G
LL

in the uninoculated broths (Fig. 53).

It was evident from these results that sulphite inhibition of meat yeasts increased with decreasing pH and was accentuated by increasing sulphite concentration. Candida vini, the non-sulphite binder, was most sensitive to these changes in pH and sulphite concentration. Acetaldehyde secretion and thereby sulphite binding by C. norvegica was apparently not a necessary requirement for detoxification of the preservative. The production of this compound, however, was influenced by cell density, pH, sulphite concentration, substrate availability and the composition of the pre-incubation medium. Thus glucose, fructose or ethanol was required for acetaldehyde production.

DISCUSSION

The present study has identified three important topics for discussion: 1) the initial level of yeast contamination (via the field and carcass) of lamb products as compared to total bacterial count; 2) the influence of sulphite on the microbial association particularly the yeast flora; and 3) the influence of pH, sulphite concentration and substrate availability on the sulphite tolerance and acetaldehyde production of meat yeasts.

1) Yeast Contamination

The present study has clearly shown that yeasts are numerically unimportant contaminants when compared to bacteria. In the field samples these organisms accounted for 0.001-2.6% vis à vis total bacterial count. Similarly, Empey and Scott (1939) isolated very few yeasts from soil (0.5%) with incubation at -1°C or (0.045%) at 20°C as compared to bacterial numbers (Table 7). Although large numbers of yeasts were associated with field samples that were extensively contaminated with bacteria, these organisms still accounted for less than 5% of the microflora.

Candida, Cryptococcus and Rhodotorula spp. were recovered from field samples in this study. These genera were noted in the literature to occur commonly in pastures. Candida famata (formerly Torulopsis candida) and Rh. rubra have been isolated from soil by di Menna (1960) and Baxter and Illston (1977). Cryptococcus albidus var. albidus was often recovered from pasture plants by di Menna (1957, 1958a, b, 1959).

The dominant yeasts in the field samples in this study were usually non-pigmented and therefore it can be assumed sensitive to damage by photodynamic effects. In Spring (March), however, 80-87% of the yeast flora of hay and soil samples were pigmented (Fig. 8a). In contrast, di Menna (1959) noted that 84-92% of the yeast flora of pasture plants in New Zealand were pigmented in late summer (February-March) although only 4-8% were pigmented in Winter (August).

The persistence of yeasts in the field cannot be accounted for in terms of resistance to damage by photodynamic effects. Indeed, only yeasts of minor importance, Rhodotorula spp. particularly Rh. rubra, were resistant to damage by photodynamic effects. Banks (1985a,b) noted that Rh. glutinis was protected from damage by photodynamic effects presumably as a consequence of the carotenoid pigments quenching singlet oxygen - ($^1O_2$) - (Sistrom et al., 1956; Cohen-Bazire and Stanier, 1958).

The low percentage of yeasts as compared to total viable count of the field samples was obtained also from the fleece and on the carcass surface. Similarly, Empey and Scott (1939) recovered few yeasts (0.26%) with incubation at -1°C or (0.02%) at 20°C from hides (Table 7). Lowry (1984) also noted that yeast numbers were less than 5% of the total microflora on lamb carcasses.

The non-pigmented yeasts dominated the yeast flora of the fleece samples in this study. Carotenoid-pigmented yeasts were only notable in October when they accounted for 40% of the flora. Although, Rhodotorula spp. are associated with coats of animals e.g. 50% of the yeast flora on hides (Empey and Scott, 1939)

representatives of this genus rarely occur on the meat surface (Empey and Scott, 1939).

Candida, Cryptococcus and Rhodotorula spp. particularly C. famata, Cr. albidus var. albidus and Rh. rubra (isolated from the field samples) were recovered from the fleece and the carcass surface. Cryptococcus laurentii, C. famata and Rh. rubra have been frequently isolated from fleece and lamb carcasses (Baxter and Illston, 1976, 1977). Lowry (1984) noted that the yeast flora of lamb carcasses consisted of Candida, Cryptococcus and Rhodotorula spp. He further noted that Candida spp. predominated in the Winter and Rh. glutinis in the Summer and correlated this with the work on pasture plants by di Menna (1959). It is noteworthy (Table 19) that Candida, Cryptococcus and Rhodotorula spp. were also isolated in this study from butchers' aprons indicating that cross-contamination via hands and equipment also occurred. From the results in this study (and those in the literature) a route of yeast contamination from the pasture to the meat product has been established.

2) Microbial Association Selected by Sulphite

Sulphite in minced lamb and lamb products inhibited the growth of pseudomonads, the dominant Gram-negative spoilage bacteria, and Enterobacteriaceae. As a consequence the slow growing yeasts and Gram-positive bacteria, Brochothrix thermosphacta and homofermentative lactobacilli proliferated. These organisms are also selected in sulphited British fresh sausages (Dowdell and Board, 1967, 1968, 1971; Banks, 1983; Dalton, 1984; Banks et al.,

1985c).

Pseudomonads inhibition is probably associated with sulphite interaction with the prosthetic group (NAD or FAD) of glucose dehydrogenase preventing gluconate formation from glucose (Hammond and Carr, 1976; Nychas, 1984). Sulphite impairs NAD^+ regeneration and the excess NADH inhibits citrate synthase activity in Enterobacteriaceae but not in Gram-positive bacteria (Weitzman, 1966; Weitzman and Jones, 1968; Banks, 1983).

Although, the level of free sulphite diminished in lamb burgers, the concentration of total sulphite remained constant and the loss of antimicrobial activity, particularly at 15°C, was attributed to the increase in bound sulphite. As the Gram-negative bacteria are not killed but remain quiescent (Banks and Board, 1982b; Nychas, 1984), the rapid reduction of the free sulphite level to below the minimal inhibitory concentrations at 15°C was associated with renewed growth of pseudomonads (at ca. 160 μg SO_2 g^{-1}) and Enterobacteriaceae (at ca. 50 μg SO_2 g^{-1}). Similar trends were observed with British fresh sausages by Banks (1983). The loss of total sulphite, however, in minced lamb was greater than in lamb burgers, the reason for this may be that the pseudoemulsion state of burgers or sausages protects sulphite from oxidation (Banks et al., 1985c, 1987).

Yeasts accounted for only a minor component (0.05-2%) when compared to total viable count in unsulphited minced lamb stored at 5°C for 6 d. This percentage reflected the proportions of yeasts to bacteria recovered from the field, fleece and carcass surface. In contrast, the reduced competition (Dalton et al., 1984) and the

sulphite tolerance of yeasts (Brown, 1977; Banks, 1983; Dalton, 1984) permitted their domination of the flora particularly in lightly contaminated sulphited minced lamb - e.g. when yeasts were 179.7% compared to total viable count after 4 d storage at 5°C. When British fresh sausages were lightly contaminated (ca. 10^4 c.f.u. g^{-1} meat) the microflora was also dominated by yeasts (Dowdell and Board, 1968; 1971; Brown, 1977; Abbiss, 1978).

Species of Candida, Cryptococcus and Rhodotorula, particularly C. famata, Cr. albidus var. albidus and Rh. rubra isolated from the field, fleece and carcass surface were also recovered from sulphited and unsulphited minced lamb and lamb products. Candida spp. however, accounted for 73% of the yeast flora of minced lamb and lamb products. The same organisms represented 60% (Nychas, 1984) and 82% (Hseih and Jay, 1984) of the yeast flora of beef. In general, therefore, the type of meat and the presence or absence of sulphite would not appear to have a profound influence on the selection of particular groups of yeasts. It is noteworthy, however, that a higher percentage of Cryptococci (21%) were isolated from lamb than from beef - 10% (Nychas, 1984). A feature noted also by Lowry (1984) and Lowry and Gill (1984) who observed that 90% of the yeast flora of lamb loins was Cryptococcus laurentii var. laurentii. Thus the yeast flora of New Zealand obviously differs from that of British lamb. The reason for this difference is not known.

The pH of sulphited lamb burgers (pH 6.25) did not change over 24 d storage at 5°C. At 15°C the growth of lactobacilli was associated with an acid drift (to pH 4.4 after 10 d storage).

Similar trends (Banks, 1983) were noted with the British fresh sausage. Sulphite has a carbohydrate or 'glucose sparing action' (Abbiss, 1978; Nychas et al., 1988) but only prevents acid formation up to a certain temperature threshold - ca. 10°C (Banks, 1983). With the rapid loss of free sulphite at 15°C, the carbohydrate sparing action was lost and the acid drift was probably associated with glucose metabolism.

The available carbohydrates (rusk, soya flour and starch) in lamb burgers are presumably made available as glucose, maltose, maltotriose and maltotetrose for microbial metabolism through the activity of α amylases and a maltase of meat origin and a β amylase from rusk (Abbiss, 1978). All the yeasts isolated from lamb products assimilated glucose, although only a few could utilise starch (3%) or maltose (44%). More importantly as regards the production of sulphite binding agents, only 14% of the meat yeasts weakly fermented glucose and none fermented maltose.

Lactate, the end-product of post-mortem glycolysis in meat (Lawrie, 1985), is also a substrate available for microbial growth (Gill, 1982; 1986). Only 47% of the meat yeasts, however, assimilated lactate. It is noteworthy that acetaldehyde is not an intermediate of lactate metabolism.

Yeasts are potentially the organisms responsible for sulphite binding in meat products. They can metabolise glucose or maltose to acetaldehyde, pyruvate and α ketoglutarate - all avid sulphite binding agents (Rankine and Pocock, 1969). Indeed, the concentration of acetaldehyde (60 μg g^{-1} meat) in lamb burgers after 6 d storage at 5°C accounted for 34% of the bound sulphite

although altogether 96% was bound. Dalton (1984) noted that the acetaldehyde concentration in sausages was positively related ($r = 0.89$, $p = <0.05$) to the amount of sulphite bound and accounted for 40% (1°C) or 70% (15°C) of the latter. As only negligible concentrations of pyruvate and α ketoglutarate were recovered from British fresh sausages (Dalton, 1984) acetaldehyde appears to be the most important even though it is not the only sulphite binding agent in sausages and lamb burgers. The identity of the other sulphite binding agents is unknown. Acetaldehyde, however, is produced by fermentation of glucose and only 14% of the meat yeasts fermented glucose.

Further evidence that yeasts are probably the only organisms that cause sulphite binding in meat products came from the work done on sausages by Dalton (1984). She noted a positive correlation between the size of the initial inoculum of Debaryomyces hansenii (the dominant yeast in sausages) and the amount of sulphite bound ($r = 0.98$ at 1°C, $r = 0.92$ at 15°C). In fact, the increase in sulphite binding associated with increased acetaldehyde concentration reflected the rate and extent of growth of yeasts in sausages particularly at 15°C (Dalton, 1984).

Sixty percent of the initial yeast flora of minced lamb were acetaldehyde producers (positive on detection medium) but as the sulphite concentration diminished with storage there was an increase in non-acetaldehyde producing yeasts. Non-sulphite binding yeasts dominated the yeast flora of vacuum-packed British fresh sausages, whereas the numbers of sulphite binding yeasts (derived from the agar plate method devised in this study) increased over

storage in commercial packs (Adams et al., 1987). Dalton (1984) noted that the sulphite binding yeasts - Deb. hansenii and C. zeylanoides - were favoured by sulphite whereas the non-sulphite binders - Rhodotorula and Cryptococcus spp. decreased in number. The differences can be accounted for by the fact that the additional carbohydrates in British fresh sausages provide glucose that can be fermented to acetaldehyde by yeasts. Sulphite binding, however, does not appear to be essential for the success of yeasts in lamb products as the preservative does not select for certain groups of yeasts, e.g. those that produce acetaldehyde.

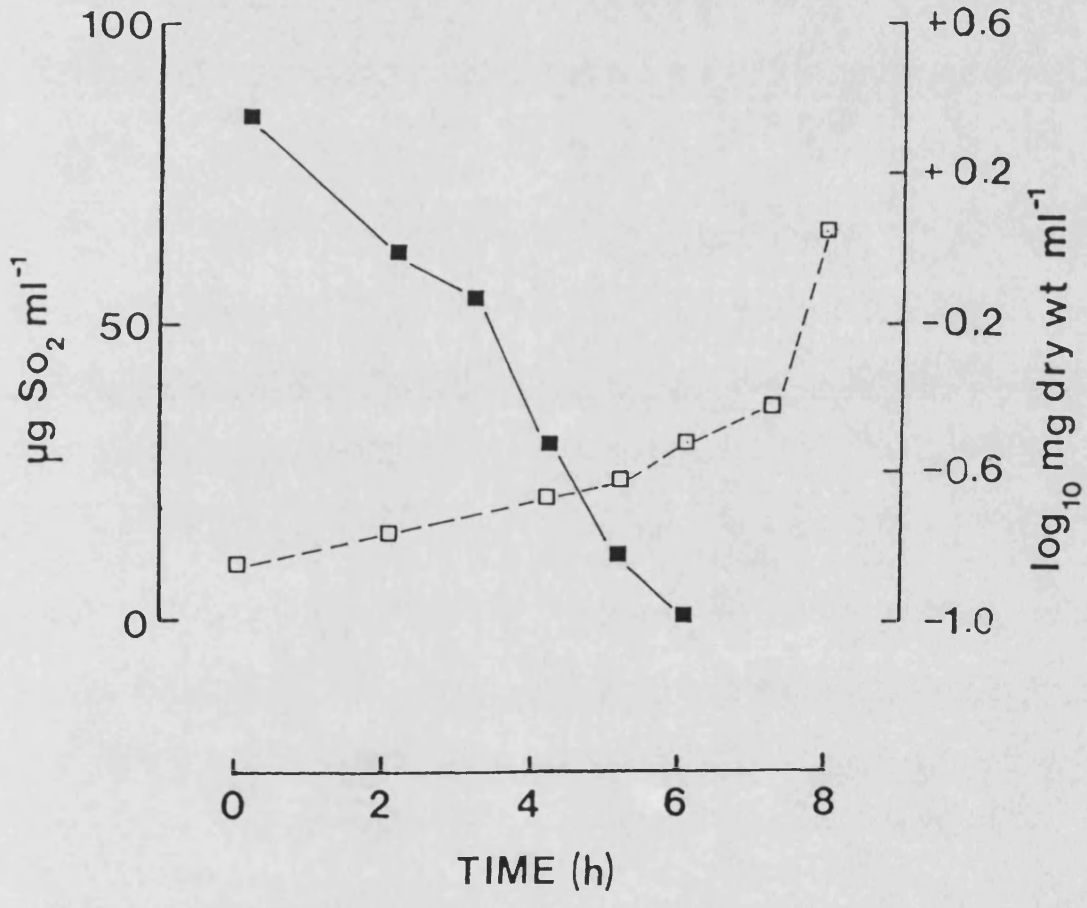
3) Sulphite Tolerance of Yeasts

Any discussion of sulphite as a preservative must stress the essential role of pH on the percentage distribution of molecular sulphur dioxide (SO_2), bisulphite (HSO_3^-) and sulphite (SO_3^{2-}) ions in aqueous solution (Hammond and Carr, 1976; King et al., 1981). The term 'sulphite' is used to denote this mixture of sulphur (IV) oxospecies. To date the effect of sulphite on yeast cells has been studied mainly with those isolated from wines and ciders of pH 2.8-4.2. Meat products have a pH of 5.8-6.8. The former therefore contains a greater percentage of molecular sulphur dioxide (Fig. 4). Sulphited meat products contain more bisulphite (HSO_3^-) and sulphite (SO_3^{2-}) ions.

Some yeasts particularly those with a fermentative metabolism are resistant to sulphite (Rehm and Wittman, 1962; Reed and Peppler, 1973). Sulphite was observed to extend the lag phase (Fig. 54) of growth of Saccharomycodes ludwigii at pH 3-5 (Stratford,

Figure 54

REDUCTION OF FREE SULPHITE CONCENTRATION AND GROWTH OF SACCHAROMYCODES LUDWIGII IN AEROBIC MEDIUM AT PH 4.0 AND 30°C.*



*Adapted from Stratford (1983)

1983) during which time acetaldehyde (with only negligible quantities of pyruvate and 2-oxoglutarate) was secreted and the resultant reduction in concentration of free sulphite was such that it was undetectable at the time that the delayed exponential growth phase commenced (Morgan, 1982; Stratford, 1983). As bound sulphite is considered not to be an antimicrobial agent (Hammond and Carr, 1976), and as acetaldehyde production was sulphite induced in S'codes ludwigii and Saccharomyces cerevisiae (Morgan, 1982; Stratford, 1983), Stratford (1983) concluded that acetaldehyde secretion was a mechanism by which these yeasts detoxified the preservative. In contrast, C. norvegica isolated from lamb burgers in this study secreted acetaldehyde (although sulphite induced) at pH 5-7 during the exponential phase of growth. This was also the case with those yeasts isolated from sausages by Dalton (1984).

The resistance of the yeast cell to the preservative may be influenced by the rate of sulphite uptake and the internal pH off the cell. Molecular sulphur dioxide is the only form of sulphite that enters the wine yeast cell over the pH range 3.0-5.0 (Macris, 1972; Macris and Markakis, 1974; Stratford, 1983; Stratford and Rose, 1986). To maintain the equilibrium, molecular sulphur dioxide drawn into the cell is replaced in solution by regeneration from the bisulphite and sulphite ions (Macris and Markakis, 1974). Macris (1972) and Macris and Markakis (1974) considered transport to be an active mediated process (sulphite permease). Morgan (1982), Stratford (1983) and Stratford and Rose (1986), however, concluded that sulphite entered the cells of Sacch. cerevisiae by passive diffusion. As the internal pH (6.5) of the yeast cell is

near neutrality (Borst-Pauwels, 1981) the concentration of molecular sulphur dioxide is lowered by conversion to bisulphite ions (Stratford and Rose, 1986) and binding to metabolic products such as acetaldehyde. This results in the yeast cell accumulating 20-30 times the external sulphite concentration to equilibrate with the external molecular sulphur dioxide concentration (Stratford, 1983). A lower internal pH, however, would reduce the amount of sulphite accumulated (Stratford, 1983; Rose, 1987) and could account for the slow diffusion rate of sulphite that enhanced the resistance of S'codes ludwigii (Stratford, 1983).

Many yeasts, particularly those that tend to have oxidative metabolism, are inhibited by the preservative. Candida norvegica and C. vini isolated from sulphited lamb burgers in this study, were inhibited by >100 and <100 μg SO_2 ml^{-1} respectively at pH 4 and the latter by >250 μg SO_2 ml^{-1} at pH 5 in lab lemco glucose broth cultures. Sulphite is a highly reactive molecule and reacts with substrates and essential growth factors and vitamins such as ascorbic acid, vitamin B_{12} and vitamin K (Taylor et al., 1986). More importantly on entering the cell, sulphite interferes with the physiology of the organism by interacting with enzymes and nucleic acids, and preventing energy production.

Sulphite causes the depletion of adenosine triphosphate - ATP (Hinze and Holzer, 1986; Prakash et al., 1986) by:

1) increasing the rate of ATP utilisation. The ionisation of molecular sulphur dioxide inside the cell liberates protons. Energy (ATP) is expended to export these protons to maintain the internal pH (Warth, 1977, 1985) and to re-establish the proton gradient.

2) Sulphite activates an ATP-hydrolysing enzyme which rapidly decreases the ATP level (Schimz and Holzer, 1979; Schimz, 1980) and 3) Sulphite prevents ATP generation from glycolysis by inhibiting the activity of glyceraldehyde-3-phosphate dehydrogenase (Hinze and Holzer, 1986). The resultant ATP depletion causes irreversible cell damage after exposure to sulphite for 1 h (Schimz and Holzer, 1979).

Sulphite inhibits enzyme activity by:

1) disruption of the secondary and tertiary structure by cleavage of the disulphide bonds e.g. glutathione reductase (Massey and Williams, 1965). Sulphite replaces the sulphur group of cystine, converting half the residues to cysteine and half to S-cysteine sulphonate (Clark, 1932; Cole, 1967).

2) Reaction with co-enzymes. Sulphite reacts with reduced and oxidised nicotinamide adenine dinucleotide (NAD^+) thus inhibiting NAD dependent reactions in glycolysis and the tricarboxylic acid (TCA) cycle (Rehm, 1964; Wedzicha, 1984).

3) Sulphite reacts with prosthetic groups and cofactors: pyridoxal phosphate (Adams, 1969), folate and dihydrofolate (Vonderschmitt et al., 1967), flavin (Wedzicha, 1984) isoalloxazines (Muller and Massey, 1969; Hevesi and Bruice, 1973) and thiamine pyrophosphate (Williams, 1935; Williams et al., 1935; Fry et al., 1957; Haisman, 1974).

In addition to inhibiting the enzymes, sulphite further disrupts glycolysis by forming addition complexes with the intermediate compounds (Hammond and Carr, 1976). Thus the formation of the acetaldehyde-bisulphite complex (hydroxysulphonate) in

anaerobic cells of Sacch. cerevisiae prevents reoxidation of NADH by ethanol dehydrogenase as acetaldehyde is not available as a hydrogen acceptor (Gancedo et al., 1968; Stratford, 1983). The shortage of NAD^+ prevents the flow of intermediates through glycolysis by inhibiting the activity of glyceraldehyde-3-phosphate dehydrogenase (Stratford, 1983). Sulphite enhanced glycerol synthesis (Neuberg and Reinfurth, 1918, 1919; Freeman and Donald, 1957a,b) is caused by the resulting accumulation of glyceraldehyde-3-phosphate. No ATP is produced but some NAD^+ is regenerated. In aerobic cells, however, glycolysis was maintained as indicated by pyruvate decarboxylase activity, regardless of the acetaldehyde-sulphite complex as NAD^+ was probably regenerated by using oxygen as an electron acceptor (Stratford, 1983).

Sulphite forms addition complexes with the pyrimidine bases, uracil and cytosine of nucleic acids (Hayatsu et al., 1970; Hayatsu and Miura, 1970; Shapiro et al., 1970). Robakis (1980) suggested that the uracil sulphonate adduct interacts with the ribosome, stopping translation of mRNA or causing the release of defective polypeptides, and thus directly inhibiting RNA and protein synthesis in Escherichia coli. The uracil-sulphonate adduct also interferes with DNA polymerase activity (Kai et al., 1974). The conversion of the cytosine-bisulphite adduct to uracil is mutagenic as the cytosine-guanine pairs between adjacent DNA strands are converted to adenine-thymine pairs (Hayatsu and Miura, 1970; Mukai et al., 1970; Shapiro, 1977). Sulphite induced mutations have been reported in bacteriophages (Hayatsu and Miura, 1970; Summers and Drake, 1971), bacteria (Mukai et al., 1970) and yeasts (Dorange and

Dupuy, 1972). The concentration of sulphite in these cases, however, was 10 to 100 fold greater than that used in beverages and foods (Rose, 1987).

As discussed so far these mechanisms of sulphite inhibition at pH 3-5 are overcome by fermentative yeasts through their ability to detoxify the preservative by the production of acetaldehyde. In contrast, yeasts that have an oxidative metabolism are sensitive to sulphite. The situation changes at pH 6-7 (the pH of meat products) when there is a greater percentage of sulphite (SO_3^{2-}) ions (5.84% at pH 6, 38.70% at pH 7) (King et al., 1981). Candida vini isolated from lamb burgers in this study was resistant to sulphite (500 µg SO_2 ml^{-1}) at pH 6-7 in lab lemco glucose or lactate broth cultures although it was an oxidative yeast and did not produce acetaldehyde. Similar results were obtained with Cr. albidus recovered from lamb burgers (Grice, 1985). Dalton (1984) also observed that the percentage of bound sulphite (23%) in broth cultures of Cr. albidus and Rh. rubra isolated from British fresh sausages, was comparable to the amount bound in the uninoculated broths and that only negligible quantities of acetaldehyde were present.

The mechanism of resistance to the preservative exhibited by C. vini is unclear but may be due to a combination of several mechanisms. Possible explanations could be: 1) the yeast cell is impermeable to sulphite (Warth, 1977; Dalton, 1984). 2) The slow transport system that might exist for bisulphite ions (Stratford and Rose, 1986) could be affected by the lipid composition of the plasma membrane (Keenan and Rose, 1979). 3) Conformation of the

sulphite receptor site on the cell surface membrane (Anacleto and van Uden, 1982). 4) Insensitivity of the cell targets to the preservative (Dalton, 1984) or modification of the sensitive sites in the cell (Warth, 1977). 5) Compensation mechanisms to overcome inhibition (Warth, 1977).

Candida norvegica, isolated from lamb burgers in this study, weakly fermented glucose and therefore presumably possessed pyruvate decarboxylase the enzyme required for pyruvate to be decarboxylated to acetaldehyde. In aerobic conditions, acetaldehyde secretion from the yeast cell was sulphite induced and occurred during the exponential phase of growth. Similar results were obtained with C. lipolytica isolated from lamb burgers (Grice, 1985). Acetaldehyde production was also sulphite induced and occurred during the exponential phase of growth in unbuffered (poised at pH 7) lab lemco glucose broth cultures of Deb. hansenii, P. membranaeaciens, C. zeylanoides and T. candida (now reclassified as C. famata) isolated from sausages (Dalton, 1984). Although her yeasts were oxidative they exhibited fermentative metabolism in sulphited (500 μg SO_2 ml^{-1}) broth which led Professor Rose (Pers. Comm.) to conclude that they possessed pyruvate decarboxylase (Dalton, 1984). As discussed earlier, this observation contrasts with wine yeasts, that secrete acetaldehyde during lag phase and do not commence exponential growth until all the free sulphite is bound. It has to be stressed, however, that with wine yeasts these observations were noted at pH 3-5.

In this study C. norvegica produced acetaldehyde in sulphited (500 μg SO_2 ml^{-1}) lab lemco glucose, fructose or ethanol broth

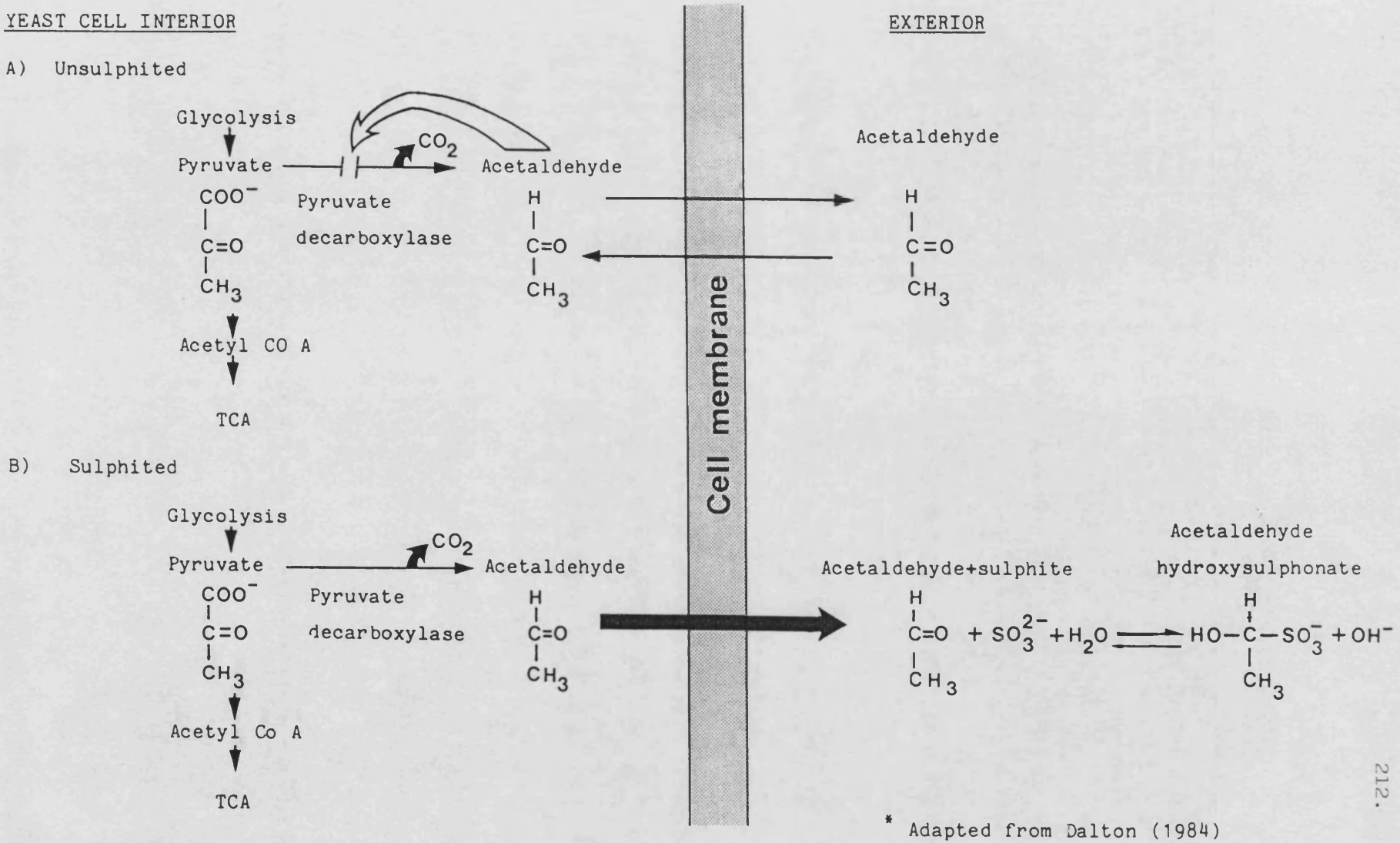
cultures buffered at pH 5, 6 or 7. At pH 7, and to a certain extent at pH 6, resistance was enhanced by the fact that a proportion of the sulphite was oxidised to sulphate. It is noteworthy that the percentage of sulphite (SO_3^{2-}) ions increases at pH 6 (5.84%) and pH 7 (38.70%) (King et al., 1981) and may be the cause for the increased loss of the preservative by oxidation.

Fructose enters glycolysis by phosphorylation via fructose-1-phosphate to glyceraldehyde-3-phosphate and like glucose is metabolised to acetaldehyde via pyruvate.

Candida norvegica in this study secreted maximum levels of acetaldehyde at pH 6 in sulphited lab lemco glucose (207.1 $\mu g\ ml^{-1}$) and fructose (175.30 $\mu g\ ml^{-1}$) broth with 74.60% and 69.90% of the sulphite bound respectively. Assuming that binding of acetaldehyde to sulphite was 1:1 (Burroughs and Sparks, 1973a), acetaldehyde accounted for the majority of the sulphite bound. Similarly, Dalton (1984) noted that in unbuffered cultures of Deb. hansenii, P. membranaefaciens, C. zeylanoides and T. candida, acetaldehyde accounted for most of the sulphite bound (94-98%). She noted that only negligible quantities of pyruvate and α ketoglutarate were present. Acetaldehyde appears to be the most important although not the only sulphite binding agent produced by meat yeasts. The identity of the other sulphite binding agents is unknown.

Assuming that acetaldehyde is exported from the cell (Fig. 55 - Dalton, 1984) the formation of the acetaldehyde-bisulphite complex (hydroxysulphonate) would disturb the equilibrium between the intra and extracellular concentration of free acetaldehyde. Consequently, acetaldehyde would diffuse out of the cell, lowering the

Figure 55 SULPHITE INDUCED ACETALDEHYDE SYNTHESIS IN MEAT YEASTS - A PROPOSED MECHANISM *



* Adapted from Dalton (1984)

intracellular concentration and more acetaldehyde would be produced until the intra and extracellular balance was restored, e.g. when all available free sulphite was bound (Dalton, 1984). Under these conditions glucose is probably being metabolised by respiro-fermentation (Käppeli, 1986) partly catabolised by respiration (electron acceptor oxygen) and partly by fermentation (electron acceptor acetaldehyde). In cells exhibiting respiro-fermentation there is a decreased activity in the enzymes of the respirative pathway (Käppeli, 1986) which steers the metabolism towards fermentation. In Sacch. cerevisiae this has been noted to occur in conditions of excess glucose (4-5% w/v) when the oxidative pathway is saturated (Käppeli, 1986).

It is evident that glycolysis was maintained in cells of C. norvegica because acetaldehyde was produced, therefore the activity of glyceraldehyde-3-phosphate dehydrogenase was not inhibited by the lack of NAD^+ (Wedzicha, 1984). As acetaldehyde was not available as a hydrogen acceptor because it was bound to sulphite (Gancedo et al., 1968; Sols et al., 1971; Stratford, 1983). NADH could not be reoxidised by ethanol dehydrogenase and NAD^+ was probably regenerated using oxygen as an electron acceptor, as observed in aerobically grown cells of Sacch. cerevisiae (Stratford, 1983). The thiamine pyrophosphate cofactor of pyruvate decarboxylase can be cleaved by sulphite (Haisman, 1974) but in this instance the enzyme is either resistant to sulphite or the preservative does not enter the cells of C. norvegica. Additionally, there may be a partial inhibition of the NAD^+/NADH dependent enzymes, the pyruvate dehydrogenase complex (pyruvate→

acetyl CoA) and ethanol dehydrogenase (acetaldehyde⟶ethanol) thus steering the cycle to overproduction of acetaldehyde.

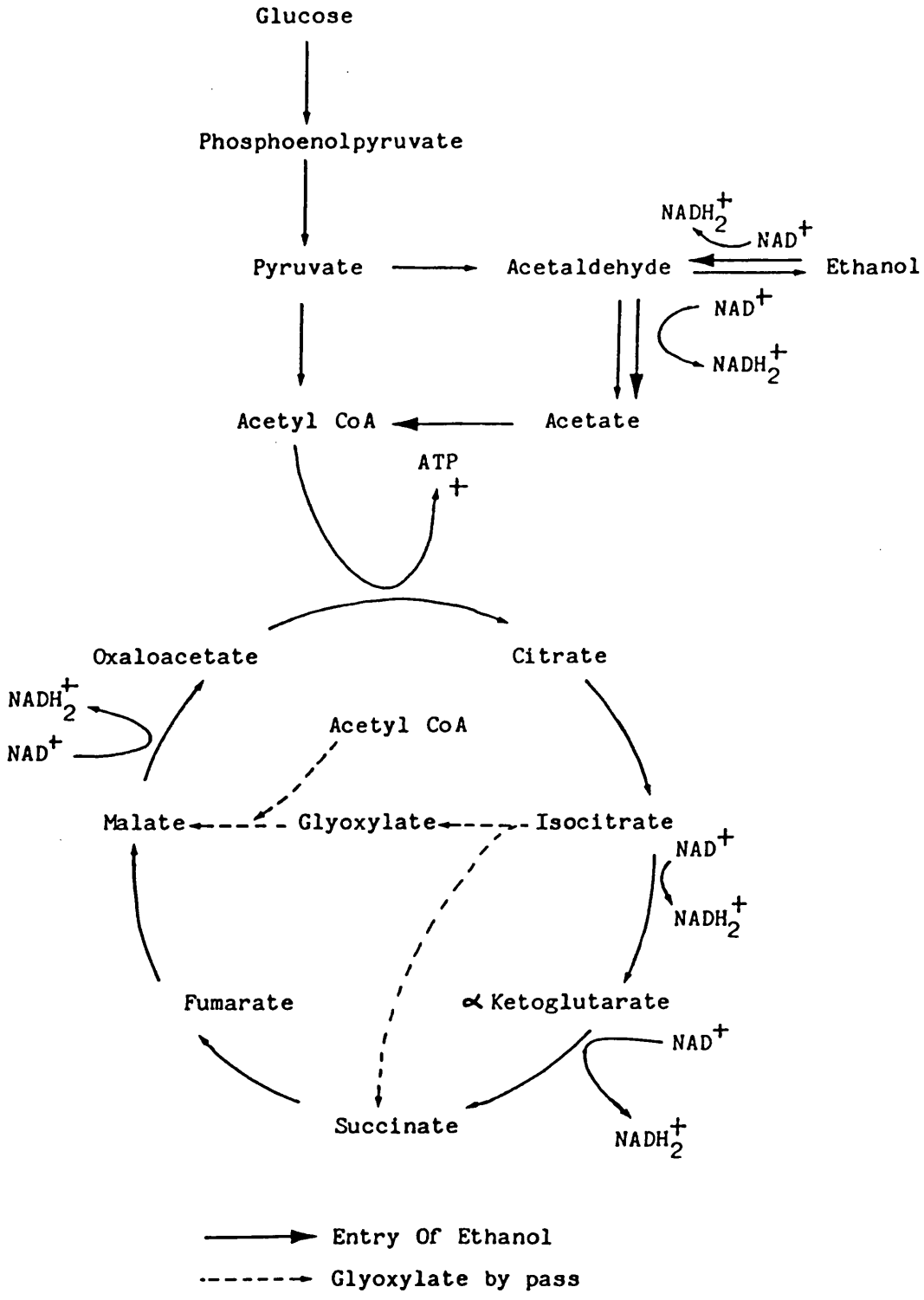
Candida norvegica produced acetaldehyde in sulphited (500 µg SO_2 ml^{-1}) lab lemco ethanol broth cultures buffered at pH 5, 6 and 7. The amount of acetaldehyde produced was far in excess of that required to bind the available sulphite. The maximum production of acetaldehyde was at pH 5 (508.80 µg ml^{-1}) and 98.65% of the sulphite was bound. The first product of ethanol oxidation in Sacch. cerevisiae is acetaldehyde (Fig. 56). The NAD^+ is utilised and $NADH_2^+$ is used in the respiratory chain (Pons et al., 1986). Regeneration of NAD^+ is therefore brought about by using oxygen as an electron acceptor (Stratford, 1983). Again the reaction may be steered towards the acetaldehyde-hydroxysulphonate to maintain the free acetaldehyde concentration.

Candida norvegica was able to grow, albeit not as well, in sulphited (500 µg SO_2 ml^{-1}) lab lemco lactate broth buffered at pH 5, 6 and 7 without secreting acetaldehyde. In fact, in glucose broth cultures of C. norvegica growth occurred before acetaldehyde was secreted, as production of this compound did not occur until the exponential phase of growth. It appears that acetaldehyde production is unnecessary for growth of this yeast and is therefore not required to detoxify the sulphite.

In the yeast cell, lactate is initially oxidised to pyruvate (Sols et al., 1971). An increase in pyruvate concentration, in the absence of sugars, enhances the activity of the enzymes of the TCA cycle and causes a decrease in pyruvate decarboxylase activity in the cells of Sacch. cerevisiae (Polakis and Bartley, 1965). Thus in

Figure 56.

METABOLISM OF ETHANOL *



* Adapted from Pons et al. (1986).

cells of C. norvegica, lactate would be oxidised to acetyl CoA via pyruvate and enter the TCA cycle. Acetaldehyde production would not occur due to the decrease in the pyruvate decarboxylase activity.

Candida norvegica grew, albeit poorly, without secreting acetaldehyde in lab lemco broth buffered at pH 6 supplemented with various substrates. Resistance to sulphite was enhanced by: 1) the fact that 20-80% of the sulphite was irretrievably lost, presumably by oxidation to sulphate; or 2) immediate binding of sulphite (89.9%) to the substrate as with pyruvate.

Glucose (and therefore fructose) is the only compound fermented by C. norvegica (Kreger-van Rij, 1984) which would account for the lack of acetaldehyde production (with the exception of ethanol) in the presence of other substrates. Notably, acetate is oxidised to acetyl CoA and enters the TCA cycle (Fig. 56 - Pons et al., 1986) and therefore would not yield acetaldehyde. The activity of pyruvate decarboxylase is decreased in the presence of pyruvate (Polakis and Bartley, 1965) and again acetaldehyde would not be produced.

When C. norvegica was transferred from a lab lemco broth to a carbohydrate enriched, sulphited (500 μg SO_2 ml^{-1}) broth buffered at pH 5 sulphite inhibited growth and acetaldehyde was not produced (Figs. 40, 52). The switch from a broth with a non-carbohydrate carbon source to one enriched with glucose or fructose means that the enzymes of gluconeogensis (e.g. fructose 1,6-biphosphatase) need to be switched off and those of glycolysis (e.g. phospho-fructokinase) activated (Gancedo, 1971; Lenz and Holzer, 1980). Initially the cyclic action of phosphofructokinase and fructose

1,6-bisphosphatase wastes ATP (Lenz and Holzer, 1980). After 3 min however, fructose 1,6-bisphosphatase and other gluconeogenic enzymes are inactivated by phosphorylation (Lenz and Holzer, 1980; Mazón et al., 1982a, b); irreversible proteolytic degradation not occurring until after 1 h (Funayamo et al., 1980). Hence ATP would not be available, for at least the first 3 min, to expel the protons liberated by ionisation of molecular sulphur dioxide (Warth, 1977, 1985) and maintain the pH of the cell. Additionally, no glycolytic products such as acetaldehyde would be present to bind the sulphite. It is noteworthy that the concentration of molecular sulphur dioxide (0.058%) at pH 5 is ten times greater than that (0.006%) at pH 6 (King et al., 1981) and may account for the greater sensitivity to sulphite at pH 5, exhibited in this case.

When C. norvegica was transferred from a lab lemco broth to a sulphited (500 µg SO_2 ml^{-1}) lab lemco ethanol broth buffered at pH 5, sulphite inhibited growth and acetaldehyde was not produced. Less degradation of gluconeogenic enzymes occurs with ethanol than with glucose (Funayamo et al., 1980) the reduction in ATP, however, may be sufficient to prevent proton efflux. Additionally, the enzyme responsible for oxidation of ethanol to acetaldehyde (Pons et al., 1986) may need to be activated, and in that case the latter would not initially be available to bind sulphite.

The mechanisms of sulphite resistance of C. norvegica and C vini would be better understood if it were known whether or not sulphite enters the yeast cells. The absence or presence of uptake could be studied by using radioactively labelled sulphite. To

further the understanding of the resistance mechanism of C. norvegica, production of ethanol and carbon dioxide in sulphited lab lemco glucose broth cultures, could be studied.

It is evident from the above discussion, that the availability of an energy (ATP) source, such as glucose, is an important factor in the resistance of the yeast cell to sulphite. Thus the energy pump (ATP) eliminates the protons accumulated intracellularly from the ionisation of molecular sulphur dioxide (Warth, 1977, 1985) and the pH of the cell is maintained. Additionally, the metabolism of glucose by glycolysis yields acetaldehyde, the major sulphite binding compound. Glucose further enhances acetaldehyde production by causing an increase in the activity of the enzymes of the anaerobic pathway and a decrease in the enzyme activity of the TCA cycle (Polakis and Bartley, 1965).

Glucose is also important in determining the onset of spoilage of meat under aerobic chill storage (2-7°C) as the breakdown of amino acids does not occur until the former is depleted. The depletion of glucose is partially due to the conversion of glucose to gluconate by the dominant spoilage bacteria, the pseudomonads (Whiting et al., 1976). The addition of sulphite, however, inhibits pseudomonads by interacting with glucose dehydrogenase thereby preventing gluconate formation (Nychas, 1984). The resultant reduction in competition favours the growth of the slow growing Gram-positive bacteria and yeasts. Consequently, the 'glucose sparing action' of the preservative (Abbiss, 1978) is removed by the sulphite binding to acetaldehyde produced by yeasts thus permitting glucose metabolism by spoilage bacteria.

To extend the shelf-life of sulphited meat products an additional preservative method could be used in conjunction with sulphite. Sorbate when used in combination with sulphite was noted to suppress the growth of lactobacilli and prevent the growth of yeasts for 7 d (Banks, 1983). By retarding the growth of yeasts the free sulphite (the antimicrobial proportion) could be maintained for a longer length of time.

APPENDIX

Classification of Yeast Species

Yeasts species noted in the literature		Yeast species as reclassified by Kreger-van Rij (1984)	
Brettanomyces spp.		Brettanomyces spp.	
Bullera alba		Bullera alba	
	tsugae		tsugae
Candida	albicans	Candida	albicans
	blankii		blankii
	bovina		pintolopesii (IMP)
		Saccharomyces telluris (P)	
	brumptii	Candida	catenulata
	buffonii		buffonii
	catenulata		catenulata
	ciferrii	Stephanoascus ciferrii	
	curvata	Candida	curvata
	diddensiae		diddensiae
	diversa		diversa
	famata		famata (IMP)
		Debaryomyces hansenii (P)	
	foliorum	Candida	foliorum
	glaebosa		glaebosa
	guilliermondii		guilliermondii (IMP)
		Pichia guilliermondii (P)	
	humicola	Candida humicola	
	iberica		zeylanoides
	ingens		ingens (IMP)
		Pichia humboldtii (P)	
	insectamans	Candida insectamans	
	intermedia		intermedia
	krusei		krusei (IMP)
		Issatchenkia orientalis (P)	
	lambica	Candida	lambica
	lipolytica	Saccharomycopsis lipolytica (P)	
		Candida lipolytica (IMP)	
	lipolytica var.		
	lipolytica	Saccaromycopsis lipolytica (P)	
	lipolytica var.		
	deformans	Candida deformans	
	melinii		melinii (IMP)
		Hansenula canadensis (P)	
	mesenterica	Candida mesenterica	
	parapsilosis	Candida parapsilosis	
	pelliculosa	Hansenula anomala (P)	
		Candida pelliculosa (IMP)	
	ravautii		catenulata
	rugosa		rugosa
	sake		sake
	scottii	Leucosporidium scottii (P)	
		Candida scotti (IMP)	
	silvae		silvae

Yeasts species noted in the literature	Yeast species as reclassified by Kreger-van Rij (1984)
silvicultrix	silvicultrix
steatolytica	steatolytica
tropicals	tropicalis
valida	valida (IMP)
	Pichia membranaefaciens (P)
vini	Candida vini
zeylanoides	zeylanoides
Cryptococcus albidus	Cryptococcus albidus (IMP)
	Filobasidium floriforme (P)
albidus var. aerius	Cryptococcus albidus var. aerius
albidus var. albidus	albidus var. albidus
albidus var. diffluens	albidus
diffluens	albidus
dimennae	dimennae
gastricus	gastricus
hungaricus	hungaricus
infirmo-miniatus	Rhodosporidium infirmominiatum (P)
	Cryptococcus infirmo-miniatus (IMP)
laurentii	laurentii
laurentii var. flavescens	laurentii
laurentii var. laurentii	laurentii var. laurentii
laurentii var. magnus	magnus
luteolus	luteolus
macerans	macerans
neoformans var. uniquttulatus	uniquttulatus (IMP)
	Filobasidium uniquttulatum (P)
skinneri	Cryptococcus skinneri
terreus	terreus
uniquttulatus	uniquttulatis (IMP)
	Filobasidium uniquttulatum (P)
Debaryomyces hansenii	Debaromyces hansenii
kleockeri	hansenii
nicotianae	hansenii
subglobosus	hansenii
marama	marama
Geotrichum candidum	Geotrichum candidum
Hanseniaspora vineae	Hanseniaspora vineae (P)
	Kloeckera africana (IMP)
Hansenula anomala	Hansenula anomala (P)
	Candida pelliculosa (IMP)
canadensis	Hansenula canadensis (P)
	Candida melinii (IMP)
polymorpha	Hansenula polymorpha
subpelliculosa	subpelliculosa

Yeasts species noted in the literature	Yeast species as reclassified by Kreger-van Rij (1984)
Kloeckera spp.	Kloeckera spp.
Leucosporidium capsuligenum	Filobasidium capsuligenum (P)
	Candida japonica (IMP)
scotti	Leucosporidium scotti (P)
	Candida scotti (IMP)
Pichia etchellsii	Pichia etchellsii
farinosa	farinosa
fermentans	fermentans (P)
	Candida lambica (IMP)
media	Pichia media
membranaefaciens	membranaefaciens (P)
	Candida valida (IMP)
vini var. melibiosi	Pichia carsonii
vini var. vini	carsonii
Rhodotorula aurantiaca	Rhodotorula aurantiaca
crocea	aurantiaca
flava	Cryptococcus flava
glutinis	Rhodotorula glutinis (IMP)
	Rhodosporidium diobovatum (P)
	sphaerocarpum (P)
	toruloides (P)
glutinis var. rubescens	Rhodotorula glutinis (IMP)
graminis	graminis
macerans	Cryptococcus macerans
marina	Rhodotorula minuta
minuta	minuta
minuta var. minuta	minuta
minuta var. texensis	minuta
mucilaginosa	rubra
pallida	minuta
rubra	rubra
texensis	minuta
Saccharomyces acidifaciens	Zygosaccharomyces bailii
cerevisiae	Saccharomyces cerevisiae
dairensis	dairensis
Kloeckerianus	Torulaspora globosa
Schizoblastosporium starkeyi-henricii	Schizoblastosporium starkeyi-henricii
Sporobolomyces odorus	Sporidiobolus salmonicolor
pararoseus	pararoseus
roseus	roseus
salmonicolor	salmonicolor
Torulopsis aeria	Cryptococcus albidus
albida	albidus
candida	Candida famata (IMP)
	Debaryomyces hansenii (P)
dattila	Kluyveromyces thermotolerans (P)
	Candida dattila (IMP)

Yeasts species noted in the literature	Yeast species as reclassified by Kreger-van Rij (1984)
domercqii	Wickhamiella domercqii (P)
	Candida domercqii (IMP)
famata	Candida famata (IMP)
	Debaryomyces hansenii (P)
glabrata	Candida glabrata
globosa	Citeromyces matritensis (P)
	Candida globosa (IMP)
gropengiesseri	gropengiesseri
holmii	holmii (IMP)
	Saccharomyces exiguus (P)
inconspicua	Candida inconspicua
inconspicua var. inconspicua	inconspicua
ingeniosa	ingeniosa
norvegica	norvegica
pseudaeria	Cryptococcus albidus
silvatica	Candida silvatica
vanderwaltii	vanderwaltii
versatilis	versatilis
Trichosporon cutaneum	Trichosporon cutaneum
pullulans	pullulans
variable	Pichia burtonii (P)
	Candida variabilis (IMP)

(P) = Perfect state
(IMP) = Imperfect state

First mentioned is the name under which the yeast is classified.

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REVIEW

Glucose, the Key Substrate in the Microbiological Changes Occurring in Meat and Certain Meat Products

G. J. NYCHAS,* VIV. M. DILLON,† AND R. G. BOARD†

*Ministry of Agriculture, Institute of Food Technology, Lycovrisi 14123, Athens, Greece, and †School of Biological Sciences, University of Bath, Bath BA2 7AY, United Kingdom

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The literature dealing with the role of glucose in the microbiological changes of meat and certain meat products is reviewed. Discussion is centered on two aspects. First, glucose plays a part in the selection of the dominant spoilage organisms, *Pseudomonas fragi, Ps. lundensis,* and *Ps. fluorescens,* on red meat stored aerobically under chill (2–7°C) conditions. It is concluded that the pseudomonads flourish because they convert glucose to the less commonly used substrate, gluconate. The latter serves as an extracellular energy store. With its depletion, the pseudomonads utilize amino acids, thereby producing the characteristic off-odors of spoiled meat. Storage of meat in a modified atmosphere (viz., 20% CO_2:80% O_2) selects Gram-positive flora (lactobacilli and *Brochothrix thermosphacta*) which impart a "cheesy odor" through acid production from glucose and volatile fatty acids from amino acids. The first mentioned organisms produce the same off-odors in "acid" meat (pH 5.5) from which oxygen is excluded. So too does the less acid-tolerant *Br. thermosphacta* in less acid meat (pH > 5.8), especially if trace amounts of O_2 are present. Such meat may be colonized by *Shewanella putrefaciens* also, with green discoloration resulting from the release of H_2S from amino acids. The addition of glucose and NO_2^- to, and the exclusion of oxygen from, comminuted meat selects a flora dominated by *Lactobacillus* spp. and staphylococci such as *Staphylococcus carnosus.* Second, sulfite, the preservative of British-style sausages, has a sparing action on glucose. As a consequence of its curtailed breakdown there is only a meager acid drift with storage even though a fermentative flora of lactobacilli and *Br. thermosphacta* is selected. Yeasts also contribute to the microbial association in sausages; members of four of the six commonly occurring genera bind sulfite through acetaldehyde production. Glucose appears to be essential for acetaldehyde synthesis. The role of glucose in spoilage and the conditions which select particular groups of spoilage organisms are considered in the context of chemical probes and/or instrumental methods for routine assessment of the "freshness" of meat and meat products.

Our forefathers had four main strategies to thwart microbial breakdown of red meat. Consumption within a short while of an animal being killed was probably the most common. For those living near polar regions, low winter temperatures permitted extended storage of an autumn catch. Depending upon climate and availability of salt, "drying" of meat by salting, smoking, or insolation could be practiced. This method curtails the growth of all but the very slow growing xerophytic fungi. The fourth method, fermentation, evolved presumably from empirical observations which led to practices conducive to the growth of Gram-positive, facultatively anaer-

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obic bacteria (1). So too did a relatively recent innovation, the use of sulfite to preserve British-style sausages (2).

Large-scale shipment of refrigerated carcasses over long distances together with an increasing availability of both commercial and domestic refrigerators in the period between the two world wars led to the recognition of Gram-negative aerobic bacteria as important spoilage organisms (3). The rapid growth of supermarkets, with a resultant need for prolonged storage of perishable commodities, immediately following the Second World War confirmed the importance of such organisms (4). Indeed their dominance in meat spoilage was preeminent until the advent of modified-atmosphere storage and vacuum packaging (5) of primal cuts and a range of meat products. The flushing of previously vacuum-packed meat or meat products with CO_2 is also advocated for long-distant shipment (6, 7). These changes led to the renewed importance of facultatively anaerobic bacteria, the majority being Gram-positive, but now as spoilage organisms rather than as contributors to the preservation of fermented sausages.

Food microbiologists have tended to follow in the wake of technological innovation in the meat industry. As is evident in the next section, their cause was not helped by inadequate taxonomic schemes. Rapid progress in molecular biology has led to major reviews of many groups of bacteria (8, 9). Indeed, revisions in nomenclature and classifications together with insights into the phylogeny of bacteria have reached an important stage. For the first time microbiologists can with confidence collaborate with biochemists in studies of the chemical changes occurring in meat spoilage. The reverse is true also in that the biochemistry underlying the conversion of living muscle to meat is now generally well characterized (10, 124). Collaborative studies are needed so that new methods of preservation can be explored and, perhaps more importantly, instrumental methods and chemical probes can be considered for routine use in novel approaches to quality control of meat processing. The available evidence emphasizes the role of muscle glycogen of the animal awaiting slaughter in the post-slaughter events which determine the fate of meat or meat products. In the discussion of this theme, an extensive literature has been surveyed. To avoid overburdening the text with a large number of primary references, reviews have been quoted when pertinent, and tables have been used to summarize information on specific topics.

MICROORGANISMS ON MEAT

Flora of marked phylogenetic and hence physiological diversity are carried on the hide and feet of cattle from fields, sheep from folds, and pigs from sties. During transit and retention in lairages, fecal material often fouls the coats of animals—some of the organisms contained therein may carry plasmids which confer resistance to a range of clinically important antibiotics (11). There is abundant evidence (12, 13) of the transfer of organisms from both sources as well as from the gut under conditions of poor butchering to the surface of freshly cut meat. The extent of transfer is apparently little affected by the amount of capital invested in an abattoir (14). Washing of a carcass at the end of the initial butchering process can diminish significantly the extent of contamination, especially if hot water (52°C) and a "sanitizer" such as acetic acid are used (152).

Spoilage Bacteria

Little purpose would be served by cataloguing the large number of genera of bacteria, yeasts, and molds which have been isolated from freshly butchered meats (e.g.,

TABLE I
The Microbial Associations That Develop on Red Meats and Certain Meat Products[a]

Preservative system	*Organisms selected hr*[b]	*Refs.*
Joints of meat with storage at 2–7°C		
Normal atmosphere	Pseudomonads and acinetobacters	(15, 16)
Modified atmosphere (CO_2 included as preservative)	*Brochothrix thermosphacta* and lactic acid bacteria	(5, 17–19)
Vacuum-packed[c]	Lactic acid bacteria, *Br. thermosphacta*,[d] Enterobacteriaceae, and *Shewanella putrefaciens*[e]	
With CO_2 added		(6)
Minced red meat with storage at 2–7°C		
Normal atmosphere	Pseudomonads and acinetobacter	
CO_2 added to atmosphere	*Br. thermosphacta* and lactic acid bacteria	(20)
Starch and sulfite added	*Br. thermosphacta,* lactic acid bacteria, and yeasts	
Vacuum produced and CO_2 added		(7)
British-style sausages[f] at 2–7°C	*Br. thermosphacta,* lactic acid bacteria, and yeasts	(2)

[a] For discussions of cured meat, see Gardner (21, 22) and Tompkins (23) and of fermented sausages see (1, 24).

[b] For details of taxonomy, see Table II.

[c] Meat is sealed in plastic bags of low gas permeability and a vacuum > 700 mm Hg is drawn. The respiration of the muscle converts O_2 to CO_2; hence, the storage atmosphere is made up of the latter and N_2 (16). For a discussion of the chemistry of this product, see (137).

[d] Through production of H_2S, this acid-intolerant organism causes a green discoloration of high-pH (≥6.0) vacuum-packed dry dark firm meat (see text for details).

[e] This organism's growth is favored by pH ≥6.0.

[f] This commodity, which contains sulfite, has a limited shelf life at chill temperatures (2). Fermented sausages have a long shelf life (24) due initially to low pH (produced by carbohydrate fermentation by lactobacilli), the addition of NO_2^-, anoxia, and subsequently to low a_w. Staphylococci, for example, *Staphylococcus carnosus* (25), and possibly other lipolytic bacteria contribute to flavor through breakdown of fats (1).

Ref. (13)). The physiological diversity of this initial contamination can be appreciated simply by considering those organisms which are selected by various storage methods (Table I). It is evident from this table that "traditional" methods for the short-term preservation of red meats—chilled (2–7°C) storage in a normal atmosphere—selects a microbial association dominated by Gram-negative, aerobic bacteria. Modifying the storage atmosphere, by using carbon dioxide as a preservative, for example, selects an association of Gram-positive, facultatively anaerobic bacteria. Similar microflora develop in fermented sausages as well as in British-style sausages containing sulfite as a preservative. These observations pose a fundamental question: what are the physiological/biochemical attributes which lead to the enrichment of the organisms listed in Table I? This topic is discussed below.

Taxonomy of Spoilage Organisms

It is evident from Table II that the taxonomy of bacteria associated with the spoilage of red meats has attracted considerable attention in the past decade. This is especially the case with the Gram-negative bacteria which dominate the microbial associations of red meats stored in a normal atmosphere at chill (2–7°C) temperatures. It is

TABLE II

Psychrotrophic[a] Bacteria Associated with Chilled Meats and Meat Products

Family/species	*Comments*	*Refs.*
Gram-negative bacteria—Aerobes		
Neisseriaceae[b]		
Psychrobacter immobilis	Oxidase-positive members of this family; resemble moraxellae; some strains form acid (aerobically) from glucose; some strains probably identified with *Achromobacter*[c] in early studies.	(38, 39, 148)
Acinetobacter spp.	Oxidase-negative members of this family; many strains show marked nutritional versatility; many contain an aldose dehydrogenase and form gluconic acid; some strains probably identified with *Achromobacter*[c] in early studies; 12 hybridzation groups (genospecies) among 85 *Acinetobacter* strains included in a recent study.	(40)
Pseudomonadaceae		
Pseudomonas[d] rRNA homology Group 1 *Pseudomonas fluorescens* Biovars I, II, III, IV, V (includes 7 clusters) *Ps. lundensis, Ps. fragi*	Oxidase-positive, glucose-oxidizing organisms with marked nutritional versatility. The order of species is the inverse of the incidence of individual species on meat.	(26–29, 41–44)
Gram-negative bacteria—Facultative anaerobes		
Vibrionaceae *Shewanella putrefaciens*[e]	More commonly a spoilage organism on fish and poultry than on red meat. Vacuum packaging of low-acid (pH ≥6.0) red meats is associated with growth of this organism; H_2S production from amino acids causes greening (16).	(45)
Enterobacteriaceae[f]	Many members of this family have been isolated from meat works and meat, viz, *Enterobacter, Serratia, Hafnia, Citrobacter.* Some species, e.g., *Enterobacter liquefaciens* grow on vacuum-packed meat of pH 6.05.	(13)
Gram-positive bacteria—Facultative anaerobes		
Catalase reaction-weak *Brochothrix thermosphacta*[g]	L-(+)-Lactic acid formed from anaerobic and acetoin, isobutyric and isovaleric acids from aerobic utilization of glucose. Enzymes of TCA cycle are almost totally absent. Growth factors required.	(59)

TABLE II—*Continued*

Catalase reaction-negative *Lactobacillus*[h] *Carnobacterium*	An inadequately studied group of acid-tolerant bacteria notable for absence of TCA cycle—hence, dependent upon exogenous sources of amino acids—and requirement for growth factors and a fermentation substrate, viz, glucose, from which lactic acid (homofermentative) or CO_2 and lactic and acetic acids (heterofermentative) are formed. The following species are commonly found on meat products: *Lactobacillus sake, divergens, carnis* and *bavaricus, farciminis, alimentarius, viridescens, curvatus.*	(31, 63–66, 158–160)

[a] For a review of the definitions of psychrophile and psychrotroph and the biochemical attributes of these two groups of bacteria, see Gounot (46).

[b] A study of inter- and intragenetic similarities of ribosomal ribonucleic acid cistrons indicated that this family contains at least five unrelated groups (47). One group containing *Acinetobacter, Moraxella,* and misnamed achromobacters is somewhat related to organisms belonging to rRNA superfamily II which contains the *Pseudomonas fluorescens* complex. See (148) for further discussions of rRNA cistron similarities among members of this family.

[c] Hendrie *et al.* (48) proposed that *Achromobacter* be considered as a *nomen dubiem.* Recently the genus has been reinstated to accommodate isolates from clinical sources (49).

[d] The pioneering study in the modern era of *Pseudomonas* taxonomy (50) did not include the organisms *Ps. fragi* and *Ps. lundensis,* of common occurrence on chilled meat, a feature first noted by Davidson *et al.* (51). A recent taxonomic study (138) has shown that *Ps. fluorescens* and *Ps. fragi* were the dominant organisms in milk stored at 5°C. Another recent study (52) has proposed the transfer of *Pseudomonas acidovorans* and *Ps. testosteroni* to the genus *Comamonas* and another (149) has proposed the erection of a new genus, *Flavimonas,* for *Pseudomonas*-like species from human clinical specimens.

[e] This organism has suffered from numerous changes in name, viz., *Pseudomonas putrefaciens* (53), *Achromobacter putrefaciens* (54), and *Alteromonas putrefaciens* (55). Van Landschoot and De Ley (56) concluded from a study of the rRNA cistrons that the genus *Alteromonas* was very heterogeneous and noted that the cistrons of *Alt. putrefaciens* resembled those of Vibrionaceae. Their findings were reflected in the study by MacDonnell and Colwell (45) who proposed that this species be renamed *Shewanella putrefaciens* and who recommended a revision of the definition of Vibrionaceae. This family and Enterobacteriaceae are included in the gamma subdivision of eubacteria by Woese *et al.* (57).

[f] *Enterobacter agglomerans,* a species occurring in unsulfited British-style sausage (67), has been the subject of a recent taxonomic study (68).

[g] This organism, which was originally isolated from American pork sausages (30), was named *Microbacterium thermosphactum* by McLean and Sulzbacher (58). The currently accepted name was proposed by Sneath and Jones (59). It is an important spoilage organism of vacuum-packed sliced meat products (60). Confusion of this organism with lactobacilli and streptococci is possible if incorrect procedures for the catalase test are used (61). It must be stressed that *Br. thermosphacta* exhibit a growth cycle (long rods → short rods → coccobacilli) during growth in the laboratory (61). A similar growth cycle is found in two occasional contaminants of meat, *Kurthia zopfii* and *K. gibsonii* (62). Motility and a strong catalase reaction distinguish the latter from the former.

[h] Members of this genus tend to be associated with radurized meat (63).

now well established that, in order of importance, the following are associated with spoilage under such conditions (26–28): *Pseudomonas fragi, Ps. lundensis,* and, normally to a lesser extent, *Ps. fluorescens,* especially strains of certain clusters of Biovar V. All these organisms belong to the *Pseudomonas* rRNA homology Group 1 (29). Somewhat related organisms such as *Acinetobacter, Moraxella,* and misnamed achromobacters occur also. Although these organisms have been isolated repeatedly from red meats, they are normally subordinate members of a spoilage flora. It is noteworthy that, because of the use of iced water to chill carcasses, the levels of contamination on freshly dressed as well as spoiled poultry by these organisms and other psychrotrophs are often greater than those on beef, pork, or lamb (16). *Shewanella putrefaciens*—formerly known as *Pseudomonas putrefaciens* or *Alteromonas putrefaciens* (Table II)—is less acid tolerant than members of the *Pseudomonas* genus. Its numbers increase in the vacuum storage of dark firm dry meat having pH > 6.0 (Table I).

Of the Gram-positive bacteria associated with red meats, the importance of *Brochothrix (Microbacterium) thermosphacta* as a spoilage organism has grown with the advent of modified-atmosphere storage and vacuum packing of meat of pH > 5.8. It is selected also by sulfite in British-style sausages (2) and by as yet uncharacterized mechanisms in American pork sausages (30). The catalase-negative, Gram-positive rods isolated from meat are assigned to the genus *Lactobacillus* (e.g. (31)). This genus is in need of major taxonomic revision (David Collins, personal communication) and little is known about the phylogenetic relationships and hence the comparative physiology of the meat isolates.

Many of the catalase-negative, rod-shaped bacteria isolated from vacuum-packed meat differ from "classical" *Lactobacillus* spp. in their inability to grow on acetate media. A recent study of these atypical lactobacilli (158) led to the definition of a new genus, *Carnobacterium,* to which *Lactobacillus divergens* (Table II) was transferred. As *Lactobacillus carnis* (Table II) was shown (158) by DNA–DNA hybridization and biochemical tests to be highly related to *Lactobacillus piscicola* (159), these species should be reduced to synonyomy with the epithet *piscicola* having priority over *carnis.*

The taxonomy of yeasts isolated from meat and meat products (32) has begun to attract attention (33) even though such organisms rarely if ever become numerically dominant in spoilage associations (34). Through being many times larger than bacteria, however, the biomass formed by yeasts may bring about chemical changes out of all proportion to the numbers in which they occur in meat products (32). Such a situation is discussed in the section on British-style sausages.

Selection of Spoilage Bacteria

Since the publication of the classic review in which Mossel and Ingram (35) defined food microbiology as a branch of microbial ecology, the selection of spoilage flora has been considered in the context of four interacting systems (36, 37): (i) the nutrient status of a food; (ii) the physiological attributes of spoilage organisms; (iii) extrinsic factors—temperature, gaseous composition of a storage environment, relative humidity, etc.; and (iv) processing factors. It is obvious from Table III that typical adult mammalian muscle has the nutritional potential to support the growth of a nutritionally diverse range of microorganisms, the potential perhaps being diminished somewhat (Table IV) in post-rigor beef, for example, because of the depletion of some nutrients (viz., glucose) and the development of an acid (pH 5.5) reaction (Fig. 1).

TABLE III

Gross Chemical Composition of Typical Adult Mammalian Muscle after Rigor Mortis[a]

Component	*Wet % weight*
Water	75.0
Protein	19.0
Lipid	2.5
Carbohydrate	1.2
Miscellaneous soluble nonprotein substances	
Nitrogenous	1.65
Inorganic	0.65
Vitamins	Trace amounts

[a] Derived from Lawrie (10). Gross chemical changes in meat composition are not associated with the development of rigor, a major event in the change from muscle to meat. The major changes are associated with low-molecular-weight soluble components to which glycolysis makes a major contribution (Table IV).

The literature of 20 or so years ago (69) leaves the impression that, simply because of the large amounts of substrate in muscle (Table III), proteolytic activity would be a priori a prerequisite of meat spoilage bacteria. This contention was bolstered also by the characteristic odors at the time of spoilage. It is now recognized that bacterial proteolysis is a late, spatially superficial, and relatively unimportant event in meat spoilage (70). The concept of proteinase involvement must take into account also factors controlling the induction of such enzymes in bacteria. Harder's (71) model assumes (i) that organisms produce very low basal levels of extracellular enzymes in the absence of an inducer, and (ii) that the regulation and extracellular production of proteinases are based on induction and end product and/or catabolite repression.

TABLE IV

The Main Low-Molecular-Weight Components of Beef Pre- and Post-Rigor Mortis[a]

	Concentration (mg/g)[b]	
Component	*Pre*	*Post*
Creatine phosphate	3.0	—
Creatine	4.5	6.5
Adenosine triphosphate	3.0	—
Inosine monophosphate	0.2	3.0
Glycogen	10	1.0
Glucose	0.5	0.1
Glucose 6-phosphate	1.0	0.2
Lactic acid	1.0	9.0
pH	7.2	5.5
Amino acids	2.0	3.5
Carnosine, anserine	3.0	3.0

[a] Based on (10, 16, 104, 124).
[b] See Fig. 1 for further details.

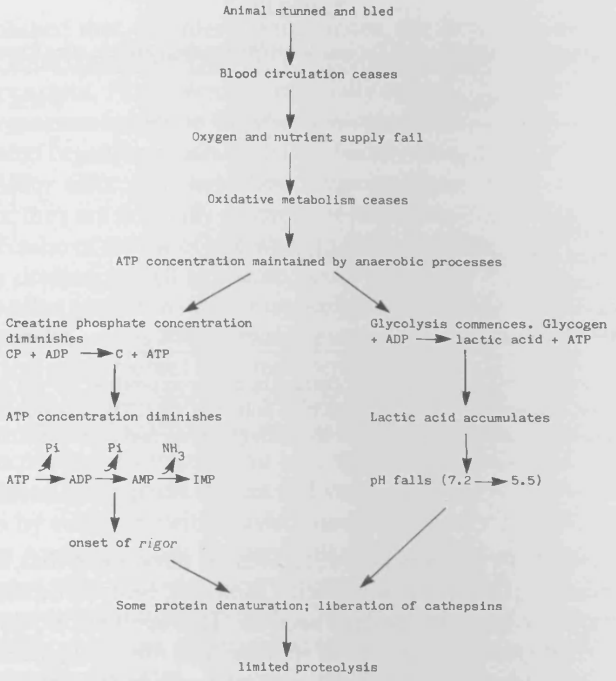

FIG. 1. Changes occurring in muscle tissue after death. Reproduced from (15) with permission.

With a clinical isolate of *Pseudomonas aeruginosa,* for example, proteinase in a glucose medium was not produced unless an inorganic nitrogen source was replaced by an organic one, glutamic acid and glutamine being the most effective inducers (72). In continuous culture, a proteinase was produced by *Ps. fluorescens* under carbon but not under nitrogen limiting conditions (73). This was taken as evidence that proteinase induction ensured that an energy rather than a nitrogen source was available to the organisms. Juffs (74) observed that glucose but not the nonutilizable lactose inhibited proteinase production by a milk isolate of *Ps. fluorescens* growing in peptone broth, the inhibition being most marked at 5°C. In view of these observations, the emphasis once accorded to microbial proteinases and their possible involvement in the breakdown of polymers in meat can, on reflection, be considered naive, a view in accord with that of Dainty *et al.* (5).

Modern methods of slaughter and butchering ensure that the freshly cut surface of meat is, at least initially, sparsely contaminated—ca. 10^3 to 10^4 bacteria per cm^2 (16). The very small proportion of psychrotrophic bacteria among the initial contaminants, 1–10%—and hence their spatial separation—obviously minimizes competition between organisms at least in the early stages of chill storage. The available evidence highlights a common feature of the spoilage organisms (Table V), their capacity to grow rapidly and hence to colonize quickly a freshly created niche in which the preferred energy source, glucose, is readily available (16). As is evident in the following sections, pO_2, pCO_2, pH, and, in some instances, antibiotic production will select the relatively few organisms (Tables I and II) present in the initial contamination that become dominant during the storage of meat and certain meat products at chill

TABLE V

Important Physiological Attributes of Principal Meat Spoilage Bacteria[a]

Attributes	*Growth rates (h) at*[b] *2°C Aerobic (anaerobic)*	*10°C Aerobic (anaerobic)*
A. Simple nutritional requirements		
1. Obligate aerobes—*Pseudomonas*	7.6	2.8
Marked nutritional diversity. Glucose preferred substrate. Maximal ATP production from available substrate. High-affinity uptake of substrate. "External storage" of energy source.[c] Little if any secondary metabolism.[d] Intolerance of CO_2 and sulfite[e,f]	(—)	(—)
2. Facultative anaerobes—Enterobacteriaceae	11.1	3.5
Relatively simple nutrient requirements ATP production determined by pO_2. Glucose preferred substrate; TCA cycle complete.	(55.7)	(8.5)
Incomplete TCA cycle—*Brochothrix thermosphacta*.[g] Glucose used (see Table VII) Tolerant of sulfite and CO_2[f].	12.0 (32.8)	3.4 (9.7)
B. Complex nutritional requirements.		
Facultative anaerobes *Lactobacillus*	—	—
TCA cycle absent. Tolerant of acid conditions, sulfite and CO_2[f]. Fermentative substrate (e.g., glucose) needed for growth. Antibiotics produced by some.	(8.4)	(4.6)

[a] For details on taxonomy, see Table II.
[b] Based on (16).
[c] See Fig. 5.
[d] The nonproduction of iron chelators such as pyoverdine may confer a competitive advantage on *Ps. fragi* over *Ps. fluorescens* in an iron-rich milieu such as meat.
[e] Enfors *et al.* (139).
[f] Banks *et al.* (2).
[g] Dainty *et al.* (105).

temperatures. In ecological terms these bacteria are opportunistic colonizers having only an ephemeral role in the events leading to the ultimate fate, mineralization, of cadavers. In what is effectively a very unstable niche, they grow rapidly at the expense of the simple, soluble nutrients which diffuse to the surface of meat. They cause, moreover, little change in the bulk chemical composition of meat even when climax populations have formed.

Such changes as do occur are sufficient to render meat unacceptable to most would-be consumers. Indeed it must be recognized that spoilage is a highly artificial—a man-defined—event in the process of meat mineralization. It is probably for this reason that its definition has been cursed by semantics (for a discussion of this issue, see Gill (16) and Jay (75)).

The situation discussed above has long been recognized by mycologists. They have given the name ruderal to fungi that initially colonize substrates, such as living plant and animal tissues, having a discontinuous nutrient input—but plentiful in soluble carbohydrate—and harboring at the outset sparse, nonresident (i.e., nonspecialized) flora (92). Such fungi are notable for their abbreviated growth phase and high repro-

TABLE VI

Bacterial Utilization of Low-Molecular-Weight Components of Meat under Aerobic Conditions[a]

Substrate	*Pseudomonad*		*Enterobacter sp.*	*Acinetobacter sp.*	*Brochothrix thermosphacta*[b]
	Nonfluorescent	*Fluorescent*			
Glycogen	—[c]	—	—	—	—
Glucose phosphate	—	—	#(2)	—	—
Glucose	#(2)[d]	#(1)	#(1)	—	#
Gluconate[e]	#(2)	#(2)	—	—	—
Casamino acids	#(3)	#(3)	#(3)	#(1)	+
Lactic acid	#(4)	#(4)	#(4)	#(2)	—

[a] Derived from Gill and Newton (78).
[b] This organism grows weakly on glycerol or ribose (5).
[c] Growth: good (#), weak (+), absent (—).
[d] Order of utilization.
[e] Taken from Farber and Idziak (105) and Nychas (106).

ductive potential. It would appear that this concept, and maybe even the term ruderal, could well be adopted by food microbiologists in order to draw attention to the very early stages of food spoilage.

Particular Attributes of Meat Spoilage Bacteria

Two important attributes of meat spoilage bacteria have been identified, relatively rapid growth rates at 2–7°C (Table V) and, in most instances, the use of glucose as preferred substrate (Table VI). It is well known (e.g., (76)) that when the concentration of a preferred substrate diminishes to growth-limiting levels, the organism having the greatest affinity will use a substrate at a faster rate than organisms having lower affinities irrespective of the relative growth rate potentials of the competing organisms. In the case of microorganisms growing on the surface of chilled meat in a normal atmosphere, little competition between organisms is thought to occur until relatively large populations are present (77), at which time the pseudomonads' affinity for oxygen gives them a competitive advantage over *Acinetobacter* and related organisms (78). The literature on meat microbiology fails to emphasize adequately the oxygen demands of *Acinetobacter.* Baumman (79), for example, demonstrated that very vigorous aeration is necessary if acinetobacter are to out-compete pseudomonads in enrichment cultures. Without adequate agitation, the aerotactic response of the motile pseudomonads results in the nonmotile competitors being deprived of oxygen. Under the conditions obtaining on the surface of meat, a pronounced diffusion gradient in glucose is established (Fig. 2) and, from what is discussed below, the rapid conversion of glucose to gluconate probably has an important role in reducing the hexose to growth-limiting levels. The commonly used total viable count method of monitoring colonization of a meat surface would not be expected to identify selective pressures due to the oxidation of glucose. Thus a more critical assessment of the implications of the composition of the general spoilage association, as well as "extraneous" contaminants, as analyzed by selective/differential media—an approach pioneered by Gardner (80)—is called for in future studies. Under anaerobic conditions

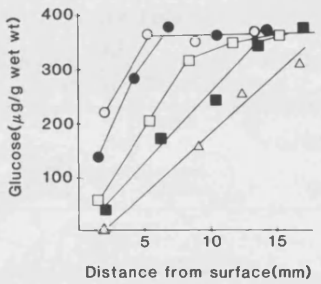

FIG. 2. Concentration gradients of glucose in meat with *Pseudomonas* sp. on the surface at viable counts per cm^2 of 2.7×10^7 (open circles), 6.3×10^7 (closed circles), 3.2×10^8 (open squares), 1.1×10^9 (closed squares), and 2.8×10^9 (open triangles). Reproduced from (16) with permission.

the success of an organism may not depend ultimately upon growth rate and its affinity for a preferred substrate. Thus with mixed cultures, *Enterobacter* sp. has been shown to outcompete *Br. thermosphacta* but not *Lactobacillus* spp. (81). In the latter case, antibiotic production by the lactobacilli may well be important. The ability of lactobacilli to inhibit the growth of other organisms is well known even though the nature of the causal agent(s) is not always clearly identified. Indeed a range of potentially toxic metabolites—lactic and acetic acids, H_2O_2—has been listed in the literature (82). Cationic polypeptides (83) may play a role in some situations also (84) and a recent study has attributed the inhibition of *Ps. fragi* to a small ($M_r \geqslant 700$) compound with an aromatic moiety produced by *Lactobacillus bulgaricus* during growth in a nutrient broth (85).

The size of the populations of pseudomonads on meat stored in a normal atmosphere is upward of 100 times larger in numbers than those of facultative anaerobes growing on meat in the absence of oxygen (Fig. 3). This presumably reflects differences in ATP production under such contrasting conditions. The ultimate size of the populations of pseudomonads is not exclusively a function of glucose/gluconate availability. Once the concentration of these falls to ca. 1 mg/g meat, inhibition of amino acid catabolism is released—an interesting example of diauxie (86)—and malodorous sulfides, esters, and acids are released (5, 87–91). Thus in this situation, the most easily detected manifestation of spoilage (off-odors) is the consequence of a

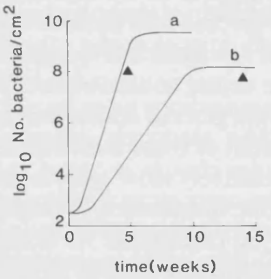

FIG. 3. Extent of microbial growth and time to spoilage (arrow) in mutton stored aerobically (a) or anaerobically (b) at 0°C. Reproduced from (15) with permission.

TABLE VII

The Effect of pH and Initial Glucose Concentration on End Product Formation by *Brochothrix thermosphacta*[a]

	Dry weight cells (mg/ml)	*Acetoin*	*Acetic acid*	*Isobutyric acid*	*Isovaleric acid*
Initial pH					
5.5[b]	1.18	3.9[c]	3.5	0.1	0.7
6.0	1.52	2.9	4.0	0.1	1.1
6.5	1.62	1.8	4.9	0.3	1.9
7.0	1.76	1.3	5.5	0.6	3.1
7.5	1.76	1.0	6.0	0.9	4.1
Glucose (% w/v)					
0[d]	0.52	0	6.3	5.5	16.7
0.06	1.12	0.7	5.8	2.7	7.6
0.40	1.63	2.7	4.9	0.4	1.6
1.00	4.20	4.7	3.4	Trace	0.1

[a] Based on (107).
[b] Growth substrate, glucose (0.2% w/v).
[c] μmol/mg dry wt.
[d] Initial pH was 6.5 and cultures were grown for 24 h.

switch in the metabolism of those organisms whose initial success in out-competing others was dependent in large part upon their preferred utilization of glucose. The growth of pseudomonads is eventually arrested not by substrate limitation but probably by the failure of oxygen to diffuse into the film of organisms developing on the surface of meat (78). Members of the Enterobacteriaceae in vacuum-packed meat grow initially at the expense of glucose also. With its depletion, amino acids are catabolized to give sulfides, amines, etc. (78, 93). In general, however, the low pH (5.5) of vacuum-packaged meats together with the progressive accumulation of CO_2 (ca. 20% v/v finally) selects Gram-positive facultative anaerobes (Table II) and off-odor production reflects the nature of the catabolism of glucose as well as that of amino acids. The lactobacilli, which produce lactic and acetic acid from glucose, impart a "cheesy" odor (94) to meat stored under anoxic conditions through volatile acid production from valine and leucine (95). The odors developing on vacuum-packed meat of pH > 5.8 are due in part to the growth of *Br. thermosphacta.* It produces lactic and, especially, acetic acids from glucose and acetoin—particularly if trace amounts of oxygen are present—as well as isovaleric and isobutyric acids from leucine and valine, respectively (61, 96–98). The actual concentrations of these products are markedly influenced by pH and the initial glucose content of the medium (Table VII). It must be stressed that the development of these malodorous substances in meats occurs at a slow rate because of the relatively slow growth rates of the causative organisms (Table V). In practice, therefore, this method of packaging extends shelf life by virtue of its selection of slow-growing organisms. As noted above, antibiotic production may play a part also in inhibiting the growth of organisms having the potential to out-compete the eventual spoilage organisms. Indeed it has been claimed that lactobacilli and *Shew. putrefaciens* produce antimicrobial agents on vacuum-packed cod

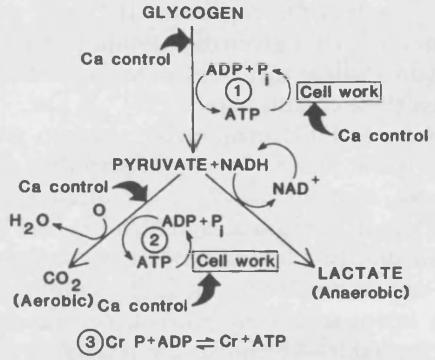

FIG. 4. Aerobic and anaerobic metabolism of muscle glycogen. ATP resynthesis from ADP plus inorganic phosphate (P_i) takes place by substrate level phosphorylation (1) and by oxidative phosphorylation (2). In addition, ATP resynthesis can occur (3) at the expense of the conversion of creatine phosphate (CrP) to creatine (Cr). This latter reaction is catalyzed by the enzyme creatine kinase and is near equilibrium in the resting cell. The points at which Ca^{2+} ions exert control over the metabolic pathways are indicated. Reproduced from (124) with permission.

and that these agents prevent the rapid growth of pseudomonads even when the fish is exposed to a normal atmosphere at the point of sell (99).

The cardinal role of glucose in determining the size of bacterial populations at spoilage and the time taken to achieve this state has been clearly demonstrated with dark, firm dry (DFD)[1] meat. Such meat is the product of many causes, of which stress, cold, and exhaustive exercise preslaughter are considered to be of particular commercial importance (10). It is now recognized that skeletal muscles of land vertebrates contain from 0.5 to 2% glycogen. Unlike liver glycogen, that of the muscle is not subject to rapid loss on fasting, providing an animal remains at rest. Hence muscle glycogen is not a food store per se. Rather it is energy stored for an emergency (151). The glycogen content of DFD is less than 50% of that of normal meat. Depletion of the residual glycogen by glycolysis produces insufficient lactic acid to cause an appreciable acid drift—pH $\geq$ 6.0 in DFD compared to pH 5.5 in normal meat (10). As little glucose occurs in DFD meat, pseudomonads catabolize amino acids early in aerobic storage and spoilage odors are rapidly produced (100). The storage life of DFD meat can be extended by inhibiting amino acid breakdown; 100 μg glucose/g of DFD extends the storage life to that of normal meat (100). Excessive addition of glucose curtails the growth of pseudomonads by creating anoxic conditions favoring the growth of lactobacilli—a feature which is exploited in the initial stages of the production of fermented sausages (24).

The immediate effects of glucose on meat spoilage cannot always be divorced from indirect ones associated with anaerobic glycolysis, which is induced by bleeding an animal (Fig. 4), occurring in the change of muscle into meat (10). The role of meat pH on the selection of *Br. thermosphacta* in vacuum-packed meat was noted above. The facultative anaerobic organism *Shew. putrefaciens* (Table II) fails to grow on meat more acid than pH 6.0. Such values occur with DFD meat which, if vacuum-

[1] Abbreviation used: DFD, dark, firm dry meat.

packed, results in the growth of this organism (101) and, through H_2S production from cysteine and serine (102, 103), green discoloration of the meat through sulfmyoglobin formation. Certain spoilage members of the Enterobacteriaceae, e.g., *Serratia liquefaciens,* grow under these conditions also (19).

The addition of glucose to DFD meat before vacuum packing does not extend shelf life. The growth of *Shew. putrefaciens* is not perturbed and that of another acid-intolerant organism, *Br. thermosphacta,* is enhanced (108). This investigation showed also that the addition of citric acid to high-pH beef before vacuum packing extended shelf life even though a lowered pH at the surface of the meat was only a transient feature. It was surmised that the acid conditions favored the growth of lactobacilli to such an extent that *Shew. putrefaciens* was unable to compete even when the pH became favorable—of course, the possibility of antibiotic production by lactobacilli or citrate having a diauxic effect ought not to be overlooked. Acid treatment of vacuum-packed lamb (pH 5.8) is essential for long-term storage; otherwise, spoilage results from the growth of *Br. thermosphacta* (109, 110).

The information given in Table V together with that presented above could well be taken as unequivocal evidence that glucose utilization, rapid growth rates, and a relative tolerance of mildly acid (pH 5.5) conditions are the main reasons for pseudomonad colonization of "normal" meat stored aerobically at 2–7°C. Recent observations suggest that a more subtle strategy underlies their success. It must be stressed that the quoted glucose content of meat post-rigor (Table IV) refers to the bulk sample examined. In practice, spoilage is confined, at least initially (69), to the surface of pieces of meat. Some limited penetration at or near the time of the onset of spoilage may occur as a consequence of the fissuring of the meat surface.

The location of the spoilage flora, as noted previously (Fig. 2), means that the availability of glucose to the spoilage flora is consequent upon a diffusion gradient which may well eventually fail to satisfy the organisms' requirements for maximal growth rate—ca. 1 mg/g meat (111). In the case of pseudomonads, it seems to have been tacitly assumed that glucose is used as and when required. Some recent observations have challenged this contention and identified a strategy which probably enhances the competitiveness of this important group of meat spoilage bacteria.

Glucose Catabolism by Pseudomonads

Glucose metabolism by pseudomonads has been studied in considerable detail (Fig. 5). As far as can be ascertained, the principal contaminants of meat, *Ps. fragi* and *Ps. lundensis,* were not included in the studies reviewed by Lessie and Phibbs (112). Because it is evident that both species are members of the fluorescent group of pseudomonads (Table II) and that the first mentioned is grouped with *Ps. fluorescens* on the basis of analysis of tyrosine biosynthesis enzymes (113), it is reasonable to assume that glucose catabolism by meat pseudomonads is unlikely to differ markedly from that of the majority of this genus.

Two pathways are used in the production of 6-phosphogluconate, a key intermediate of the Entner-Doudoroff pathway (Fig. 5). The direct oxidation pathway acts on glucose extracellularly and the other intracellularly. These can be considered as truly alternative pathways in the production of the intermediate noted above in that one can compensate for the loss of the other. Factors selecting for one or other pathway are summarized in Table VIII. Midgley and Dawes (114) proposed that glucose and

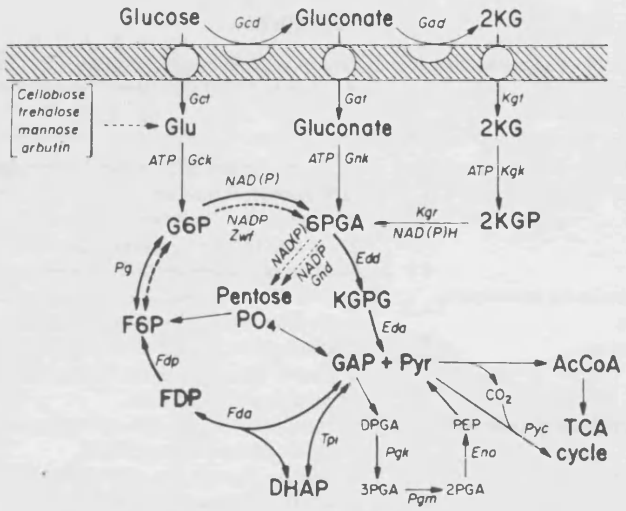

FIG. 5. Glucose metabolism by pseudomonads. Enzymes related to glucose utilization in pseudomonads. Dashed lines indicate features characteristic of *Ps. cepacia*. All other reactions shown have been demonstrated in *Ps. aeruginosa*. Sugars and intermediates are of the D-configuration. Abbreviations: Gcd and Gad, membrane-associated glucose and gluconate dehydrogenases: Gct, Gat, and Kgt and Gck, Gnk, and Kgk represent, respectively, transport systems and ATP-dependent kinases for glucose, gluconate, and 2-ketogluconate; Kgr, 2-keto-6-phosphogluconate reductase; Zwf and Gnd, glucose-6-phosphate (G6P) and 6-phosphogluconate (6PGA) dehydrogenases; Edd and Eda, 6PGA dehydratase and KDPG aldolase. Recycling of glyceraldehyde 3-phosphate (GAP) into the Entner–Doudoroff pathway involves: Tpi, triose phosphate isomerase; Fda, fructose 1,6-diphosphate aldolase; Fdp, fructose-1,6-diphosphatase; and Pgi, phosphoglucoisomerase; Pyc refers to pyruvate carboxylase. Reproduced from (112) with permission.

gluconate dehydrogenases are located in the cytoplasmic membrane and that glucose is thereby catabolized in the periplasmic space. It has been surmised that, in natural environments, the rapid oxidation of glucose may well sequester this energy source in a form, gluconate, which is not so readily utilized by organisms other than pseudomonads. Consequently it can be considered to be an extracellular storage product (115, 116). Recent studies suggest that this strategy may enhance the competitiveness of pseudomonads growing on meat under chill conditions.

Studies of microbial metabolism in a food such as meat are difficult. Basically these studies are concerned with systems, batch cultures, which are an anathema to biochemists. Not only does the physiological age of organisms vary with time but so too does the composition of the association of organisms causing spoilage. Indeed, Mossel (36) stressed the ephemeral character of the primary microflora of foods in general. Additionally the attributes of the solid "medium" change also. Some are mediated by meat enzymes (Table IV) and others by the principal as well as the subordinate members of the microbial associations. The establishment/amendment of diffusion gradients plays a role also. Two were noted previously, the outward flow of glucose to organisms colonizing the surface of meat and the inward flux of oxygen into the dense film of organisms developing at this site. Despite all these potential problems, Gardner and Stewart (117) showed that, by using selective media to monitor the composition of microbial associations, it was possible to associate bulk changes in the chemical content of meat with particular groups of organisms. When the nature

TABLE VIII

Factors Influencing Glucose Catabolism by Pseudomonads[a]

Factors	*Response*
Presence of TCA cycle intermediates—citrate, succinate, malate	Diauxie ensures use of TCA cycle intermediates before carbohydrate utilization. These do not necessarily inhibit enzymes of the direct oxidative pathway of glucose (Fig. 5)—but rather the transport of glucose—or influence the function of glucose-metabolizing enzymes.
Glucose limitation on an ammonium-limited culture	Diversion of glucose metabolism from extracellular oxidative to the intracellular phosphorylative route.
Presence of gluconate	Inhibits transport of glucose into cell and represses formation of glucose transport system. Transport of gluconate and 2-ketogluconate not affected.
Anaerobic respiration of glucose with nitrate	Gluconate and ketogluconate not formed but phosphorylative pathway intact.
Oxygen limitation	Decrease in concentration of dissolved oxygen causes a switch from extracellular (direct) oxidative pathway to the intracellular phosphorylative route (Fig. 5).
Water activity	Decrease in a_w increases glucose and gluconate dehydrogenase activity.
Temperature	Glucose predominantly oxidized and gluconate accumulates extracellularly at 5°C.

[a] Based on (112, 114–116, 118–120, 121A, B, C, 122, 141).

of the environment afforded by meat (Table IV) is set against factors (Table VIII) influencing glucose metabolism by pseudomonads, it is evident that, in addition to gross changes in substrates such as glucose, attention must be paid to the possible and probably transient occurrence of intermediates such as gluconate. This has been done in two recent studies.

Farber and Idziak (105) inoculated γ-irradiated (10 kGy) pieces of bovine longissimus dorsi muscle (3.0 × 1.5-cm blocks) with a spoilage association or pure cultures of pseudomonads, *Br. thermosphacta,* or *Enterobacter agglomerans.* As was to be expected, the growth of the spoilage flora was associated with a diminution in the glucose and lactic acid content of meat wrapped in a gas-permeable film during storage at 4°C. There was also a notable increase in the gluconate concentration by the 6th day but a marked loss of this compound by the 12th day of storage (Table IX). These workers demonstrated an increase in the concentration of glucose dehydrogenase activity throughout storage. Analysis of meat inoculated with pure cultures of bacteria revealed that gluconate occurred only with inocula of *Ps. fluorescens, Ps. putida,* or nonpigmented pseudomonads (Table IX).

Minced beef was used in the second study (20). This effectively minimized problems with glucose diffusion, thereby ensuring that spoilage organisms had immediate access to this substrate from the beginning of an experiment. Selective conditions were used to promote spoilage associations of particular composition. Sulfite alone or in combination with CO_2 was used for this purpose. From the 3rd day onward of storage of minced beef in a normal atmosphere, a progressive diminution in the glucose content of beef initially having a pH of 5.85 was demonstrated. The loss was associated with an increase in the gluconate content in the 3- to 5-day period of stor-

TABLE IX

Changes in the Concentrations of Glucose and Gluconate in Bovine Longissimus Dorsi Muscle and Minced Meat Stored in Air

Days storage	*Glucose (mg/g)*	*Gluconate (μg/g)*	*Initial pH*
0[a]	0.062	2.1	5.65
6	0.024	40.6	
12	0.002	5.8	
0[b]	0.077	10.45	5.85
2	0.073	17.00	
3	0.084	51.85	
5	0.036	98.55	
7	0.03	23.35	
9	0.02	14.50	
0[b]	0.036	2.96	6.10
1	0.064	3.45	
2	0.061	13.16	
3	0.001	6.20	
5	0.000	2.00	

[a] Taken from (105)—longissimus dorsi muscle with storage at 4°C.
[b] Taken from (106)—minced beef stored at 1°C.

age, but thereafter the concentration of the latter declined (Table IX). These trends were evident even if somewhat muted in minced beef having the characteristics of DFD meat, i.e., a relatively high pH (6.10) and a small content of glucose at the outset (Table IX). Analysis of the spoilage flora showed that pseudomonads were dominant in these two experiments. When the growth of these organisms was suppressed (Table X) by sulfite or sulfite and carbon dioxide, there was an increase in the glucose content over the first 4 days of storage with, on three occasions, a slight reduction by the 10th day. Gluconate concentration increased to a significant extent only when there was an increase in the size of populations of pseudomonads. When such changes were delayed by the preservatives, so too were the changes in gluconate concentration. These changes with "normal" glucose concentrations are illustrated in Fig. 6. With minced meat having an exceptional initial amount of glucose (Table X), gluconate concentration increased with only a small increase in the populations of pseudomonads. The meat containing preservatives supported a population of Gram-positive bacteria—*Br. thermosphacta* and uncharacterized lactobacilli. In view of the known pathways of glucose catabolism in these organisms, the failure to demonstrate even a transient accumulation of gluconate in the experiment recorded in Fig. 6 was not unexpected. The changes in the glucose dehydrogenase (Table X) content of meat supporting the growth of Gram-positive facultative anaerobes provide further circumstantial evidence that pseudomonads were the major cause of the oxidation of glucose to gluconate. Indeed these two studies support a view expressed previously: pseudomonads out-grow would-be competitors on meat by sequestering glucose as gluconate. This conclusion is of particular interest to those who seek chemical probes to assess meat freshness.

TABLE X

The Effect of Sulfite and Carbon Dioxide on Changes in the Concentration of Gluconate and Its Relationship to the Growth Rates of *Pseudomonas* spp[a] and Glucose Dehydrogenase Activity in Minced Beef at 1°C

Days		*Glucose (μg/g)*		*Growth of pseudomonads (log_{10})*		*Glucose dehydrogenase activity*		*Concentration of gluconate (μg/g)*	
	1	33.5[b]	33.5	5.1[a,b]	5.1	5.25[b]	5.25	2.96[b]	2.96
	2	63.9	68.5	5.5	5.6	4.28	3.42	3.45	3.55
A[c]	3	60.1	68.5	7.7	6.4	5.11	3.06	13.15	3.7
	4	1.0	71.5	8.3	6.7	0.72	3.7	6.2	3.4
	6	0.1	58.0	10.25	6.1	0.018	1.08	2.05	3.7
	1	11.1	11.1	4.9	4.9	3.61	3.61	8.2	8.2
A	2	39.7	30.2	5.9	5.2	2.38	1.38	19.35	7.05
	4	1.5	31.8	8.2	6.2	2.08	1.096	5.92	25.52
	5	0.0	38.1	9.5	6.0	1.07	1.26	5.85	15.7
	1	122.6	122.6	5.25	5.25		**	16.85	16.85
	2	148.4	156.1	5.45	5.2			54.75	11.8
B	3	143.9	181.6	6.85	6.0			65.26	55.7
	5	155.4	191.9	7.8	6.95			92.55	101.26
	7	137.8	207.9	7.9	6.4			243.5	101.1
	10	54.0	250.0	8.8	6.3			4.81	139.75
	1	77.15	77.15	—[d]	—		**	10.45	10.45
	2	55.7	77.15	—	—			20.6	7.15
B	3	72.8	87.15	—	—			17.0	9.85
	4	83.5	85.5	—	—			51.85	13.1
	6	35.9	80.0	—	—			98.5	11.1
	8	30.0	137.3	—	—			25.35	8.7
	10	20.0	46.0	—	—			14.5	13.3

[a] From (20).
[b] Control (no CO_2 or sulfite).
[c] A, minced meat and CO_2, B, minced beef containing sulfite, initial concentration 500 mg/kg.
[d] —, Not determined.
** Glucose dehydrogenase inhibited by sulfite (123).

Other Chemical Changes in Meat

A UK chain store has used a CO_2:O_2 gas mixture for several years to retard microbial growth in minced meat. In an extensive comparative study of minced meat from this and other sources (Table XI), Nychas and Board (20) found that during storage at 1°C the gas mixture favored the growth of Gram-positive flora of facultatively anaerobic bacteria, lactobacilli, and, to a lesser extent, *Br. thermosphacta,* whereas pseudomonads were favored in minced meat packed conventionally (Table XII). Thus the extension in shelf life of CO_2:O_2 packaged mince can be attributed to the relatively slow growth rates of the selected (CO_2-tolerant) organisms and their limited metabolism of amino acids. In the context of probes for assessing the freshness of minced meat, this comparative study highlighted marked differences in the chemical changes in the meats supporting the two contrasting microbial associations (Fig. 7).

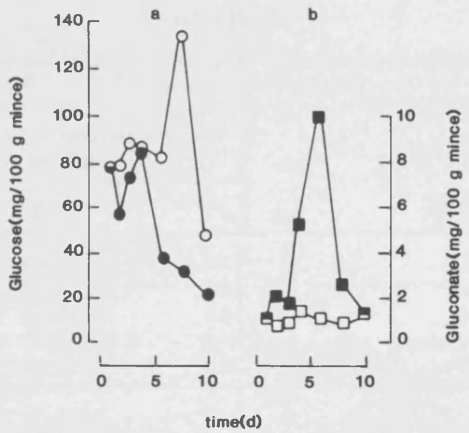

FIG. 6. Influence of sulfite concentration on the fate of (a) glucose and (b) gluconate in minced beef stored at 1°C. Minced beef with (open symbols) or without (closed symbols) sulfite. Reproduced from (2) with permission.

The growth of pseudomonads on minced meat was associated with a rapid breakdown of glucose, a marked alkaline drift (with a commensurate change in titratable alkalinity), and a rapid decrease in the extract release volume (ERV). When the growth of these organisms was suppressed, the minced meat became acid with storage, the glucose concentration built up over the first 3 days before declining at a rapid rate, and there was little if any change in the ERV. The changes in pH were to be expected in that the lactobacilli and *Br. thermosphacta* would ferment glucose to

TABLE XI

A Summary of Observations Made during a Microbiological Survey of Minced Meat[a]

		Number of organisms/g of meat packaged in various ways					
Medium	*Organism*	*A*[b]		*B*		*C*	
Plate count agar	Total viable count	7.10 (5.30–8.70)[c]	**27.46**[d]	8.50 (7.40–9.30)	**26.50**	8.80 (7.80–9.80)	**25.95**
CFC Medium	*Pseudomonas* spp.	5.80 (5.00–8.00)	—[e]	7.60 (7.0–8.50)	—	8.60 (7.70–9.30)	—
STAA	*Brochothrix thermosphacta*	4.95 (4.20–7.20)	**20.88**	6.90 (5.20–8.10)	**21.50**	8.00 (6.80–8.90)	**23.56**
Keddie's medium	Lactobacilli	4.95 (3.20–6.20)	**19.20**	6.10 (4.30–7.30)	**19.03**	5.65 (4.20–7.00)	**16.60**
VRBG	Enterobacteriaceae	4.20 (2.80–5.65)	**16.25**	5.60 (3.60–6.85)	**17.40**	5.75 (4.40–6.40)	**16.90**
Rose bengal chloramphenical agar	Yeasts	4.20 (2.80–5.60)	**16.25**	4.95 (3.70–6.00	**15.40**	5.75 (4.40–6.60)	**16.90**

[a] Taken from (106).

[b] A, Rigid plastic container, hermetically sealed with CO_2:O_2 atmosphere; B, packed on trays overwrapped with flexible transparent film; and C, meat packed at butchers' at time of purchase.

[c] 40 samples of each type examined immediately following purchase—average and range (in parentheses) given.

[d] Percentage contribution to microbial association.

[e] —, Not included in computation of contribution to microbial association because CFC medium was not used at beginning of survey.

TABLE XII

Effects of Different Methods of Packaging on the Rates of Growth of Bacteria in Minced Meat Stored at 1°C[a]

Organism	Generation times (h) in meat packed in different ways		
	A[b]	*B*	*C*
Pseudomonas spp.	37.7	14.9	15.1
Enterobacteriaceae	40.6	21.7	16.7
Lactic acid bacteria	16.0	36.1	38.0
Brochothrix thermospacta	31.0	24.1	28.1
Yeasts	26.0	21.9	23.0

[a] Taken from (106).
[b] See Table XI for details.

lactic and acetic acid (146, 147) as well as other products of an acid/neutral nature—e.g., acetoin (98A, B)—whereas the pseudomonads would oxidize the hexose to CO_2 and water before metabolizing lactic acid and amino acids. Indeed it was noteworthy that the rapid phase in the alkaline drift in minced meat supporting the growth of pseudomonads began on the day (3rd) when glucose had been effectively exhausted (106).

SULFITE-BINDING YEASTS

The contribution of yeasts to chemical changes occurring in meats preserved in various ways has been ignored so far in this discussion. Yeasts are relatively common contaminants of meat and meat products (32) but their slow rate of replication at chill temperatures rarely if ever results in their numerical dominance of spoilage associations. Walker and Ayres (34) considered yeasts to be casual contaminants of foods unless some environmental extreme—low a_w, pH, for example—favored their growth over that of bacteria. In the comparative studies of minced beef noted above (20), a CO_2:O_2 storage atmosphere impeded yeast growth, whereas the proportion of these organisms in the microbial associations of sulfited mince increased. Sulfite, as noted by Christian (125), selects a Gram-positive and hence slow-growing flora. The selective role of sulfite plays an important role also in "directing" chemical changes occurring in British-style sausages (2). Some recent evidence has identified the involvement of glucose in these changes.

From a review of the literature, Banks *et al.* (2) noted that, in addition to selecting a flora of lactobacilli, *Br. thermosphacta,* and yeasts in British-style sausages, sulfite had an apparent sparing action on glucose and was associated with the meager pH changes occurring during the storage of this product. They noted also that sulfite was a labile preservative, some being lost irretrievably at the time of sausage production and some during storage. The majority of sulfite is bound during storage. Table XIII shows that representatives of four of the commonly occurring genera of yeasts in sausages produce acetaldehyde when grown in sulfited medium. Dalton (126) demonstrated a correlation ($r = 0.89$) between the concentration of bound sulfite and acetaldehyde in British-style sausages ($n = 27$).

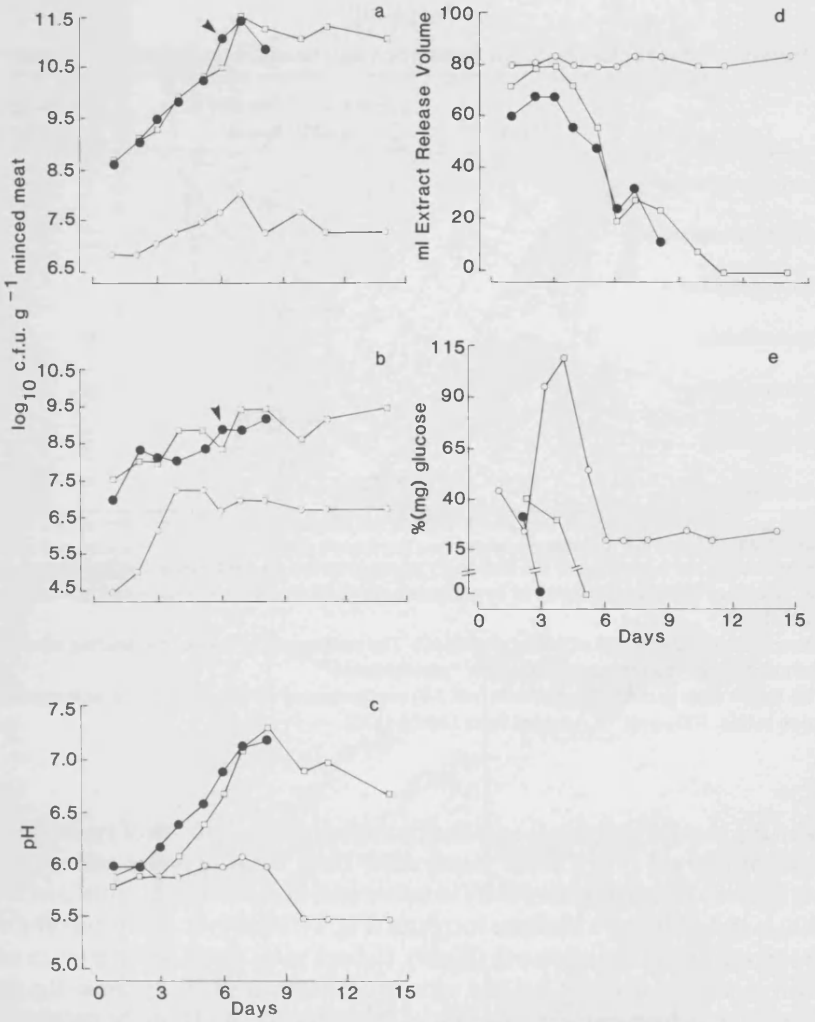

FIG. 7. Changes in microbial numbers and physicochemical attributes of minced beef packed in different ways. Open circles, minced beef prepacked in sealed semirigid plastic containers with modified atmosphere (CO_2 added); open squares, minced beef prepacked on tray and wrapped with flexible transparent film; closed circles, minced beef weighed out at counter of butcher's shop and wrapped at time of purchase. All samples stored at 1°C. Each point, mean of three observations. Arrow, time at which sample was considered spoiled. (a) *Pseudomonas* count made on the CFC medium (154); (b) count of lactic acid bacteria (155, 156); (c) pH of a 1:10 meat:water homogenate; (d) extract release volume (ERV) determined by Jay's (157) method; (e) glucose determined by SOD-period method (Boehringer-Manhein Gmbh). Taken from (106).

Of course, these observations of sulfite binding by acetaldehyde are not novel; this phemonenon has been studied extensively in acid products such as wine (127) and ciders (127, 128). With the latter, one is concerned with sulfur dioxide-tolerant yeasts, a topic discussed by Rose (129). With perhaps the most-studied representative of the spoilage yeasts of fruit juices, *Saccharomycodes ludwigii,* the antimicrobial agent is bound before exponential growth begins (130). In general, there tends to be an in-

TABLE XIII

Acetaldehyde Production and Sulfite Binding by Yeasts Isolated from British Fresh Sausages[a]

Yeast	*Broth*	*Acetaldehyde[b] (mg liter^{-1}) (% sulfite bound)*	*Percentage sulfite bound[c]*
Debaryomyces hansenii	−	11.7	—
	+[d]	178.0 (99)	98
Pichia membranaefaciens	−	13.2	—
	+	265.0 (128)	98
Candida zeylanoides	−	7.6	—
	+	138.2 (97)	94
Torulopsis candida	−	7.55	—
	+	177.2 (99)	95.5
Cryptococcus albidus	−	6.6	—
	+	8.8 (11)	23
Rhodotorula rubra	−	5.5	—
	+	7.1 (6.3)	23
Uninoculated broth	+	4.4 (−)	22

[a] Taken from Banks *et al.* (2).

[b] Determined at the beginning of the stationary phase (incubation, 30°C) by a distillation and titration method. Accuracy of method monitored by analyzing sterile broth supplemented with known amounts of acetaldehyde.

[c] Determined by the method of Banks *et al.* (140). The results quoted under this heading were obtained in experiments other than those quoted under "acetaldehyde."

[d] A lab lemco yeast extract glucose broth (pH 7.0) supplemented with sodium metabisulfite (initial concentration sulfite, 500 μg ml^{-1}). Adapted from Dalton (126).

crease in the mass of individual cells during the lag phase of growth of yeasts in sulfited medium of low pH (131). Meat yeasts differ from those of wines and ciders. The former show an increasing sensitivity to sulfite and, in particular, bisulfite and eventually SO_2 as the acidity of a medium increases (Fig. 8). Moreover, sulfite binding occurs in the exponential phase of growth (Fig. 9). Indeed, meat yeasts fail to grow in sulfited medium more acid than pH 5.0 and wine and cider yeasts fail to grow as the pH of a medium approaches neutrality. In view of the influence of pH on the proportions of SO_2, HSO_3^-, and SO_3^- in a growth medium (132), these observations have identified a little-explored and maybe a fundamental difference in the physiology of sulfite resistance of yeasts from these two sources even though acetaldehyde appears to be a common mechanism for neutralizing the antimicrobial action of sulfite.

Recent studies at Bath University have shown (Table XIV) that acetaldehyde production—and hence the potential to bind HSO_3 and SO_3 by meat yeasts—is dependent upon available substrates. Indeed, the induction of acetaldehyde production by sulfite occurred only when meat yeasts were supplied with glucose, fructose, or, more especially, ethanol, all these substrates being catabolized by pathways (Fig. 10) in which acetaldehyde is a potential intermediate (133). Another intermediate and potential binding agent, pyruvate, bound sulfite rapidly when included in a medium as a substrate (Table XIV). The growth of the meat yeasts was depressed by sulfite, the extent of inhibition being less when sulfite was bound. It is evident from these preliminary results that the influence of sulfite on yeast energetics needs to studied in detail.

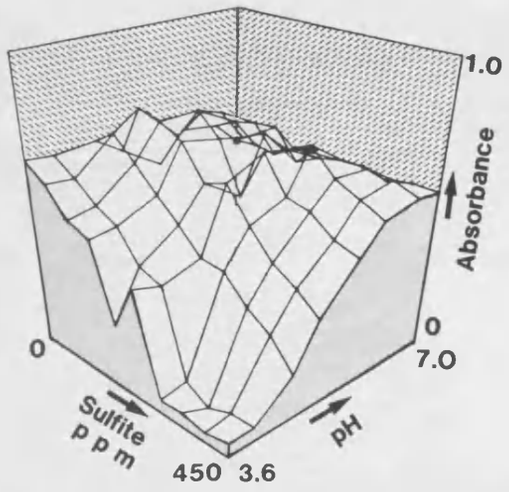

FIG. 8. The interaction of sulfite and pH on the growth of a sulfite-binding yeast. Microtiter plates were used with incubation at 25°C. Unpublished observations. The authors thank M. Cole and M. Stratford for overseeing the transformation of data.

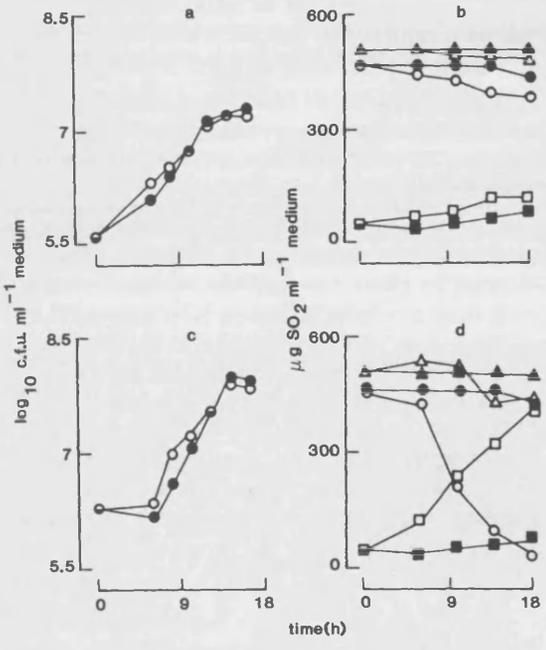

FIG. 9. Influence of sulfite concentration on the growth of (a) *Cryptococcus albidus* and (c) *Candida zeylanoides* and on the fate of free and total preservative (b) and (d) in lab lemco broth. Initial concentration of sulfite added was 500 μg ml^{-1} as determined by the method of Banks *et al.* (140). (a) and (c) ○, unsulfited; ●, sulfited broth. (b) and (d) Circles, free sulfite; squares, bound sulfite; triangles, total sulfite concentration. Open symbols, broth inoculated with yeast; closed symbols, broth inoculated with sterile distilled water. Reproduced from (2) with permission.

TABLE XIV

The Influence of Substrates at pH 6 on Sulfite Binding by a Yeast with Incubation at 25°C for 20 h[a]

Substrate[b] *(0.5 or 2% w/v)*	*Acetaldehyde*[c] *(mg liter^{-1}) (% sulfite bound)*	*Percentage bound sulfite*[d]	
Acetate	2.1 (2.6)	21.9	
Cellobiose	0.4 (0.3)	9.7	
Glycerol	1.0 (0.5)	7.3	
3-*O*-Methylglucose	1.3 (1.2)	20.1	
Lactate	2.8 (1.0)	6.0	
Maltose	3.8 (1.5)	7.6	
Salicin	0.9 (0.4)	6.5	$P < 0.1$[e]
Sorbitol	2.7 (1.5)	10.5	
Sorbose	3.0 (1.9)	13.7	
Succinate	0.5 (0.6)	31.5	
Sucrose	1.6 (0.8)	7.2	
Starch	0.5 (0.6)	17.1	
Pyruvate[f]	0.03 (0.01)	(89.9)	
Fructose	175.3 (54.6)	69.9	
Glucose	207.1 (62.7)	74.6	$P < 0.001$[g]
Ethanol	352.4 (114.8)	95.3	

[a] Yeasts grown in lab lemco peptone broth with or without specific substrates.

[b] Substrates other than ethanol sterilized at 10 psi flash in water and added aseptically to medium. Ethanol was filter sterilized.

[c] Determined by Boehringer assay kit.

[d] Determined by the method of Banks and Board (153).

[e] Comparison (in duplicate) of percentage sulfite bound in uninoculated and inoculated broth containing the listed substrate; no significant difference.

[f] Pyruvate bound sulfite to an equal extent in both inoculated and uninoculated broths.

[g] With fructose, glucose, and ethanol (4 or 5 replicates), there was a significant difference ($P < 0.001$) between sulfite bound in the inoculated and that bound in the uninoculated broth.

In the context of the microbiology of British-style sausages, it can be deduced that glucose has a key role in acetaldehyde production and hence sulfite binding. This compound is always present in substrate concentration because the farinaceous component (upward of 12% w/w) of this type of sausage is broken down throughout storage by meat amylases (134). It must be stressed that the binding of sulfite in sausages changes the environment such that Gram-negative bacteria begin to multiply once the free sulfite concentration falls below the threshold of their tolerance (2). In other words, the environment of a preserved product like a sausage is unstable—as in so many foods (36)—because of an interplay of endogenous enzymes and microbial catabolism of substrates, especially glucose.

CONCLUDING REMARKS

This review has focused attention on the role of glucose in the microbially induced changes which occur in stored meat and certain meat products. Although the slaughter of animals and the butchering/processing of meat are associated with contamina-

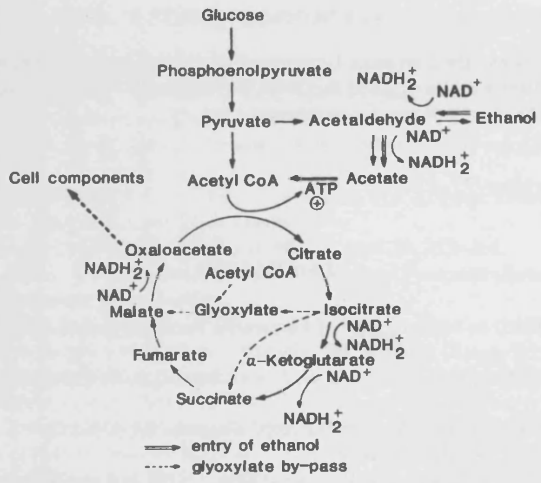

FIG. 10. Metabolic pathways of yeasts in which acetaldehyde is an intermediate. Reproduced from (133) with permission.

tion by a physiologically diverse range of microorganisms, the use of glucose as a preferred substrate appears to be a common denominator of the great majority of those which eventually contribute to the spoilage/climax populations. The physiological diversity of these organisms is reflected in the range of catabolic pathways for glucose. This raises an important question: can any one chemical or instrumental method be devised for general use in the food industry in order to assess meat freshness? In keeping with earlier reviews (135), the answer would appear to be no.

The glucose content of meat per se is probably too variable (10) to merit consideration as a general indicator of storage life potential even though demonstration of its depletion would indicate incipient spoilage especially of meat stored in a normal atmosphere at 2–7°C. The variety of potential products from glucose metabolism is probably too diverse—and their occurrence in easily detectable amounts occurs too late in storage life—to base an analytical system on end product analysis. If the preferred substrate and the products of its metabolism offer little scope, then one is left with enzyme analysis to link the two. As systems, many quantitative, based on antibodies are now in routine use for many clinical and commercial purposes—viz., identification of meat species (136)—could this technology provide another approach to an age-old problem, the routine assessment of the "freshness" of meat or meat products? Alternatively microbial sensor systems (see Ref. (150) for an introduction to the literature) may have a role to play. In a recent study (150) with fish, for example, freshness was assayed with an oxygen electrode the tip of which was capped with a membrane filter containing *Alteromonas (Shewanella) putrefaciens* and covered with dialysis membrane. A filtered homogenate of fish was pumped through a cell containing the amended oxygen electrode and the current decrease (A) recorded. The current decrease (B) produced by pumping medium through the cell was also recorded and the ratio A/B was used to express freshness. In practice the assimilation of substrates from the fish homogenate was indexed by measuring the biochemical oxygen demand of the spoilage organism.

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